

Receipt is hereby acknowledged for the following in the U.S. Patent and Trademark Office:

Applicant: Robert E. Reiter and Owen N. Witte
Title: PSCA: PROSTATE STEM CELL ANTIGEN AND USES THEREOF
Docket: 30435.54USI3
Express Mail No.: EE889993409US
Date of Deposit: May 25, 1999

Transmittal sheet, in duplicate, containing Certificate under 37 CFR 1.10.
Patent Application: Spec. 95 pgs; 23 claims (3 pgs); 1 page abstract
53 sheets of formal drawings
Verified Statement Claiming Small Entity Status
A signed Combined Declaration and Power of Attorney
A copy of the executed assignment to The Regents of the University of California, Recordation Form
Cover Sheet
A check in the amount of \$525.00 to cover the filing fee
Return postcard

Patent

SBA

EE889993409US

EXhibit 115

Receipt is hereby acknowledged for the following in the U.S. Patent and Trademark Office:

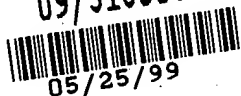
Applicant: Robert E. Reiter and Owen N. Witte
Title: PSCA: PROSTATE STEM CELL ANTIGEN AND USES THEREOF
Docket: 30435.54USI3
Express Mail No.: EE889993409US
Date of Deposit: May 25, 1999

Transmittal sheet, in duplicate, containing Certificate under 37 CFR 1.10.
Patent Application: Spec. 95 pgs; 23 claims (3 pgs); 1 page abstract
53 sheets of formal drawings
Verified Statement Claiming Small Entity Status
A signed Combined Declaration and Power of Attorney
A copy of the executed assignment to The Regents of the University of California, Recordation Form
Cover Sheet
A check in the amount of \$525.00 to cover the filing fee
Return postcard

Patent

SBA

jc542 U.S. PTO
09/318503





UNITED STATES POSTAL SERVICE™

POST OFFICE TO ADDRESSEE EE889993409US

ORIGIN (POSTAL USE ONLY)

PO ZIP Code 91103	Day of Delivery <input type="checkbox"/> Next <input checked="" type="checkbox"/> Second	Flat Rate Envelope <input type="checkbox"/>
Date In: 5-23-97	Mo. Day Year 23 03 97	Postage \$ 15.75
Time In: 2:00 PM	12 Noon <input type="checkbox"/> 3 PM <input type="checkbox"/>	Return Receipt Fee \$
Weight 13.0 lbs.	Military <input type="checkbox"/> 2nd Day <input type="checkbox"/> 3rd Day <input type="checkbox"/>	COD Fee \$
No delivery <input type="checkbox"/> Weekend <input type="checkbox"/> Holiday <input type="checkbox"/>	Int'l Alpha Country Code	Insurance Fee \$
Acceptance Clerk Initials DUC	Total Postage & Fees \$ 15.75	

CUSTOMER USE ONLY

METHOD OF PAYMENT:

Express Mail Corporate Acct. No.

Federal Agency Acct. No. or Postal Service Acct. No.

☐ **WAIVER OF SIGNATURE** (Domestic Only) Additional merchandise insurance is void if waiver of signature is requested. Merchandise to be made without obtaining signature of addressee or addressee's agent (if delivery employee judges that article can be left in secure location) and authorize that delivery employee's signature can suffice as valid proof of delivery.

☐ **NO DELIVERY** ☐ Weekend ☐ Holiday

Customer Signature

FROM: (PLEASE PRINT)

PHONE ()

Sarah B. Adriano
Daniel & Adriano
35 W. Arroyo Parkway, Suite 60
Pasadena, California 91103

OKT. 30435.54US13

TO: (PLEASE PRINT)

PHONE ()

Assistant Commissioner for
Patents
United States Patent and
Trademark Office
Washington, D.C. 20231

Box Patent Application

FOR PICKUP OR TRACKING CALL 1-800-222-1811 www.usps.gov

Label 11-B July 1997

LB0X Patent Application

PRESS HARD.

You are making 3 copies.

FOR PICKUP OR TRACKING CALL 1-800-222-1811

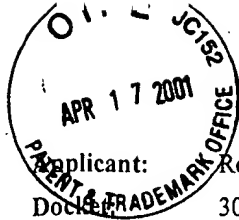
www.usps.gov



Label 11-B July 1997

Customer Copy





IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Robert E. Reiter and Owen N. Witte

Docket No.: 30435.54USI3

Title: PSCA: PROSTATE STEM CELL ANTIGEN AND USES THEREOF

CERTIFICATE UNDER 37 CFR 1.10

'Express Mail' mailing label number: EE889993409US

Date of Deposit: May 25, 1999

I hereby certify that this paper or fee is being deposited with the United States Postal Service 'Express Mail Post Office To Addressee' service under 37 CFR 1.10 and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

By: Renato Marco P. Domingo
Name: Renato Marco P. Domingo

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

We are transmitting herewith the attached:

- ☒ Transmittal sheet, in duplicate, containing Certificate under 37 CFR 1.10.
- ☒ Utility Patent Application: Spec. 95 pgs; 23 claims (3 pgs); Abstract 1 pg.
The fee has been calculated as shown below in the "Claims as Filed" table.
- ☒ 53 sheets of formal drawings
- ☒ Verified statement to establish small entity status
- ☒ A signed Combined Declaration and Power of Attorney
- ☒ A copy of the executed assignment of the invention to The Regents of the University of California, Recordation Form Cover Sheet
- ☒ A check in the amount of \$525.00 to cover the Filing Fee
- ☒ Return postcard

CLAIMS AS FILED

Number of Claims Filed	In Excess of:		Number Extra		Rate		Fee
Basic Filing Fee							\$380.00
Total Claims							
23	20	=	3	x	9.00	=	\$27.00
Independent Claims							
5	3	=	2	x	39.00	=	\$78.00
MULTIPLE DEPENDENT CLAIM FEE							\$0.00
TOTAL FILING FEE							\$485.00

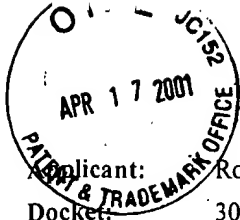
Please charge any additional fees or credit overpayment to Deposit Account No. 50-0306. A duplicate of this sheet is enclosed.

MANDEL & ADRIANO

35 N. Arroyo Parkway, Suite 60
Pasadena, CA 91103
(626)395-7801

By: Sarah B. Adriano

Name: Sarah B. Adriano
Reg. No.: 34,470
Initials: SBA



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Robert E. Reiter and Owen N. Witte

Docket: 30435.54USI3

Title: PSCA: PROSTATE STEM CELL ANTIGEN AND USES THEREOF

CERTIFICATE UNDER 37 CFR 1.10

'Express Mail' mailing label number: EE889993409US

Date of Deposit: May 25, 1999

I hereby certify that this paper or fee is being deposited with the United States Postal Service 'Express Mail Post Office To Addressee' service under 37 CFR 1.10 and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

By: Renato Marco P. Domingo
Name: Renato Marco P. Domingo

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

We are transmitting herewith the attached:

- ☒ Transmittal sheet, in duplicate, containing Certificate under 37 CFR 1.10.
- ☒ Utility Patent Application: Spec. 95 pgs; 23 claims (3 pgs); Abstract 1 pg.
The fee has been calculated as shown below in the "Claims as Filed" table.
- ☒ 53 sheets of formal drawings
- ☒ Verified statement to establish small entity status
- ☒ A signed Combined Declaration and Power of Attorney
- ☒ A copy of the executed assignment of the invention to The Regents of the University of California, Recordation Form Cover Sheet
- ☒ A check in the amount of \$525.00 to cover the Filing Fee
- ☒ Return postcard

CLAIMS AS FILED

Number of Claims Filed	In Excess of:		Number Extra		Rate		Fee
Basic Filing Fee							\$380.00
Total Claims							
23	20	=	3	x	9.00	=	\$27.00
Independent Claims							
5	3	=	2	x	39.00	=	\$78.00
MULTIPLE DEPENDENT CLAIM FEE							\$0.00
TOTAL FILING FEE							\$485.00

Please charge any additional fees or credit overpayment to Deposit Account No. 50-0306. A duplicate of this sheet is enclosed.

MANDEL & ADRIANO

35 N. Arroyo Parkway, Suite 60
Pasadena, CA 91103
(626)395-7801

By: Sarah B. Adriano

Name: Sarah B. Adriano
Reg. No.: 34,470
Initials: SBA



**APPLICATION
FOR
UNITED STATES LETTERS PATENT**

To whom it may concern:

Be it known that

Robert E. Reiter and Owen N. Witte

have invented certain new and useful improvements in

PSCA: PROSTATE STEM CELL ANTIGEN AND USES THEREOF

of which the following is a full, clear and exact description.

**PSCA PROSTATE STEM CELL ANTIGEN AND USES THEREOF**

This application is a continuation-in-part (CIP) of U.S. Serial No. 09/251,835, filed February 17, 1999, which is a continuation-in-part (CIP) of U.S. Serial No. 09/203,939, filed December 2, 1998, which is a CIP of U.S. Serial No. 09/038,261, filed March 10, 1998; claiming the priority of provisional applications, U.S. Serial No. 08/814,279, filed March 10, 1997; U. S. Serial No. 60/071,141 filed January 12, 1998 and; U. S. Serial No. 60/ 074,675, filed February 13, 1998, the contents of all of which are incorporated by reference into the present application.

Throughout this application, various publications are referenced within parentheses. The disclosures of these publications are hereby incorporated by reference herein in their entireties.

BACKGROUND OF THE INVENTION

Prostate cancer is currently the most common type of cancer in American men and the second leading cause of cancer related death in this population. In its advanced stages, prostate cancer metastasizes preferentially to bone, where it forms osteoblastic lesions. After initial treatment with androgen ablation therapy, most metastatic prostate cancers become hormone-refractory and lethal. Current diagnostic and therapeutic modalities are limited by a lack of specificity and an inability to predict which patients are at risk of developing metastatic disease.

Most prostate cancers initially occur in the peripheral zone of the prostate gland, away from the urethra. Tumors within this zone may not produce any symptoms and, as a result, most men with early-stage prostate cancer will not present clinical symptoms of the disease until significant progression has occurred. Tumor progression into the transition zone of the prostate may lead to urethral obstruction, thus producing the first symptoms of the disease. However, these clinical symptoms are indistinguishable from the common non-malignant condition of benign prostatic hyperplasia (BPH).

One of the fundamental problems in the diagnosis and treatment of prostate cancer is the lack of a marker that can accurately detect early-stage, localized tumors. Although a number of markers have been identified and some, like PSA, are in widespread clinical use, the ideal prostate tumor marker has yet to be characterized. A similar problem is the lack of an effective prognostic marker for determining which cancers are indolent and which ones are or will be aggressive. PSA, for example, fails to discriminate accurately between indolent and aggressive cancers. In addition, there is also a great need for markers that might serve as targets for therapeutic methods such as antibody-directed therapy, immunotherapy, and gene therapy.

Currently, there is no effective treatment for the 20-40% of patients who develop recurrent disease after surgery or radiation therapy or for those patients who have metastatic disease at the time of diagnosis. Although hormone ablation therapy can palliate these patients, the majority inevitably progresses to develop incurable, androgen-independent disease (Lalani et al., 1997, Cancer Metastasis Rev. 16: 29-66).

Early detection and diagnosis of prostate cancer currently relies on digital rectal examinations (DRE), prostate specific antigen (PSA) measurements, transrectal ultrasonography (TRUS), and transrectal needle biopsy (TRNB). At present, serum PSA measurement in combination with DRE represents the leading tool used to detect and diagnose prostate cancer. Both have major limitations that have fueled intensive research into finding better diagnostic markers of this disease.

PSA is the most widely used tumor marker for screening, diagnosing, and monitoring prostate cancer today. In particular, several immunoassays for the detection of serum PSA are in widespread clinical use. Recently, a reverse transcriptase-polymerase chain reaction (RT-PCR) assay for PSA mRNA in serum has been developed. However, PSA is not a disease-specific marker, as elevated levels of PSA are detectable in a large percentage of patients with BPH and prostatitis (25-86%) (Gao et al., 1997, Prostate 31: 264-281), as well as in other nonmalignant disorders and in some normal men, a factor which significantly limits the diagnostic specificity of this marker. For example, elevations in serum PSA of between 4 to 10 ng/ml are observed in

BPH, and even higher values are observed in prostatitis, particularly acute prostatitis. BPH is an extremely common condition in men. Further confusing the situation is the fact that serum PSA elevations may be observed without any indication of disease from DRE, and vice-versa. Moreover, it is now recognized that PSA is not prostate-specific (Gao et al., supra, for review).

5

Various methods designed to improve the specificity of PSA-based detection have been described, such as measuring PSA density and the ratio of free vs. complexed PSA. However, none of these methodologies have been able to reproducibly distinguish benign from malignant prostate disease. In addition, PSA diagnostics have sensitivities of between 57-79% (Cupp & Osterling, 1993, Mayo Clin Proc 68:297-306), and thus miss identifying prostate cancer in a significant population of men with the disease.

Prostate-Specific Membrane Antigen (PSMA) is a recently described cell surface marker of prostate cancer that has been the subject of various studies evaluating its use as a diagnostic and therapeutic marker. PSMA expression is largely restricted to prostate tissues, but detectable levels of PSMA mRNA have been observed in brain, salivary gland, small intestine, and renal cell carcinoma (Israeli et al., 1993, Cancer Res 53: 227-230). PSMA protein is highly expressed in most primary and metastatic prostate cancers, but is also expressed in most normal intraepithelial neoplasia specimens (Gao et al., supra). Preliminary results using an Indium-111 labeled, anti-PSMA monoclonal antibody (mAb) to image recurrent prostate cancer show some promise (Sodee et al., 1996, Clin Nuc Med 21: 759-766). Whether PSMA will prove to be a useful therapeutic target remains to be determined. However, PSMA is a hormone dependent antigen requiring the presence of functional androgen receptor. Since not all prostate cancer cells express androgen receptor, PSMA's utility as a therapeutic target may be inherently limited.

25

Clinical staging of prostate cancer is another fundamental problem facing urologists today. Currently, clinical staging relies on rectal examination to determine whether the tumor remains within the borders of the prostatic capsule (locally confined) or extends beyond it (locally advanced), in combination with serum PSA determinations and transrectal ultrasound guided biopsies. However, because of the subjectivity involved, clinical staging by DRE regularly

30

underestimates or overestimates local extension of the tumor, frequently misjudges its location, and correlates poorly with volume and extent of the tumor (Lee, C.T. and Osterling, J.E. Cancer of the Prostate: Diagnosis and Staging. In: Urologic Oncology, W. B. Saunders Company, Philadelphia, pp 357-377 (1997)). Serum PSA levels are also utilized for staging purposes, but
5 PSA alone has not been able to reliably stage prostate tumors. No technique has proven reliable for predicting progression of the disease. Thus, there is a need for more reliable and informative staging and prognostic methods in the management of prostate cancer.

SUMMARY OF THE INVENTION

10 The invention provides a novel prostate cell-surface antigen, designated Prostate Stem Cell Antigen (PSCA), which is widely over-expressed across all stages of prostate cancer, including high grade prostatic intraepithelial neoplasia (PIN), androgen-dependent and androgen-independent prostate tumors. The PSCA gene shows 30% homology to stem cell antigen-2 (SCA-
15 2), a member of the Thy-1/Ly-6 family of glycosylphosphatidylinositol (GPI)-anchored cell surface antigens, and encodes a 123 amino acid protein with an amino-terminal signal sequence, a carboxy-terminal GPI-anchoring sequence, and multiple N-glycosylation sites. PSCA mRNA expression is highly upregulated in both androgen dependent and androgen independent prostate cancer xenografts. In situ mRNA analysis localizes PSCA expression to the basal cell epithelium,
20 the putative stem cell compartment of the prostate. Flow cytometric analysis demonstrates that PSCA is expressed predominantly on the cell surface and is anchored by a GPI linkage. Fluorescent in situ hybridization analysis localizes the PSCA gene to chromosome 8q24.2, a region of allelic gain in more than 80% of prostate cancers.

25 PSCA may be an optimal therapeutic target in view of its cell surface location, greatly upregulated expression in certain types of cancer such as prostate cancer cells. In this regard, the invention provides antibodies capable of binding to PSCA which can be used therapeutically to destroy such prostate cancer cells. In addition, PSCA proteins and PSCA-encoding nucleic acid molecules may be used in various immunotherapeutic methods to promote immune-mediated destruction of
30 prostate tumors.

PSCA also may represent an ideal prostate cancer marker, which can be used to discriminate between malignant prostate cancers, normal prostate glands and non-malignant neoplasias. For example, PSCA is expressed at very high levels in prostate cancer in relation to benign prostatic hyperplasia (BPH). In contrast, the widely used prostate cancer marker PSA is expressed at high levels in both normal prostate and BPH, but at lower levels in prostate cancer, rendering PSA expression useless for distinguishing malignant prostate cancer from BPH or normal glands. Because PSCA expression is essentially the reverse of PSA expression, analysis of PSCA expression can be employed to distinguish prostate cancer from non-malignant conditions.

The genes encoding both human and murine PSCA have been isolated and their coding sequences elucidated and provided herein. Also provided are the amino acid sequences of both human and murine PSCA. The invention further provides various diagnostic assays for the detection, monitoring, and prognosis of prostate cancer, including nucleic acid-based and immunological assays. PSCA-specific monoclonal and polyclonal antibodies and immunotherapeutic and other therapeutic methods of treating prostate cancer are also provided. These and other aspects of the invention are further described below.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Nucleotide (A) and translated amino acid (B) sequences of a cDNA encoding human PSCA (ATCC Designation 209612).

FIG. 2. Nucleotide sequence of a cDNA encoding murine PSCA homologue.

FIG. 3. Alignment of amino acid sequences of human PSCA, murine PSCA, and human stem cell antigen-2 (hSCA-2). Shaded regions highlight conserved amino acids. Conserved cysteines are indicated by bold lettering. Four predicted N-glycosylation sites in PSCA are indicated by asterisks. The underlined amino acids at the beginning and end of the protein represent N terminal hydrophobic signal sequences and C terminal GPI-anchoring sequences, respectively.

FIG. 4. Hydrophobicity plot of human PSCA.

FIG. 5. Chou-Fassman analysis of human PSCA.

5

FIG. 6. A western blot showing that monoclonal antibody 1G8 binds LAPC9 (PSCA positive control) and a transitional cell carcinoma (bladder carcinoma) designated bladder (Rob).

FIG. 7. Restricted Expression of PSCA mRNA in normal and cancerous tissues. A: RT-PCR analysis of PSCA expression in normal human tissues demonstrating high expression in prostate, placenta, and tonsils. 1ng of reverse-transcribed first strand cDNA (Clontech, Palo Alto, CA) from the indicated tissues was amplified with PSCA gene specific primers. Data shown are from 30 cycles of amplification. B: RT-PCR analysis of PSCA expression demonstrating high level in prostate cancer xenografts and normal tissue. 5ng of reverse-transcribed cDNA from the indicated tissues were amplified with PSCA gene specific primers. Amplification with β -actin gene specific primers demonstrate normalization of the first strand cDNA of the various samples. Data shown are from 25 cycles of amplification. AD, androgen-dependent; AI, androgen-independent; IT, intratibial xenograft; C.L., cell line.

FIG. 8. Schematic representation of human PSCA, murine PSCA, and human Thy-1/Ly-6 gene structures.

FIG. 9. Northern blot analysis of PSCA RNA expression. A: Total RNA from normal prostate and LAPC-4 androgen dependent (AD) and independent (AI) prostate cancer xenografts were analyzed using PSCA or PSA specific probes. Equivalent RNA loading and RNA integrity were demonstrated separately by ethidium staining for 18S and 28S RNA. B: Human multiple tissue Northern blot analysis of PSCA RNA. The filter was obtained from Clontech (Palo Alto, CA) and contains 2ug of polyA RNA in each lane.

FIG. 10. Northern blot comparison of PSCA, PSMA, and PSA RNA expression in prostate cancer xenografts and tumor cell lines. PSCA and PSMA demonstrate high level prostate cancer specific gene expression. 10 µg of total RNA from the indicated tissues were size fractionated on an agarose/formaldehyde gel, transferred to nitrocellulose, and hybridized sequentially with ³²P-labelled probes representing PSCA, PSMA, and PSA cDNA fragments. Shown are 4 hour and 72 hour autoradiographic exposures of the membrane and the ethidium bromide gel demonstrating equivalent loading of samples. BPH, benign prostatic hyperplasia; AD, androgen-dependent; AI, androgen-independent; IT, intratibial xenograft; C.L., cell line.

FIG. 11. In situ hybridization with antisense riboprobe for human PSCA RNA on normal and malignant prostate specimens. A: PSCA RNA is expressed by a subset of basal cells within the basal cell epithelium (black arrows), but not by the terminally differentiated secretory cells lining the prostatic ducts (400X magnification). B: PSCA RNA is expressed strongly by a high grade prostatic intraepithelial neoplasia (PIN) (black arrow) and by invasive prostate cancer glands (yellow arrows), but is not detectable in normal epithelium (green arrow) at 40X magnification. C: Strong expression of PSCA RNA in a case of high grade carcinoma (200X magnification).

FIG. 12. Biochemical analysis of PSCA protein. A: PSCA protein was immunoprecipitated from 293T cells transiently transfected with a PSCA construct and then digested with either N-glycosidase F or O-glycosidase, as described in Materials and Methods. B: PSCA protein was immunoprecipitated from 293T transfected cells, as well as from conditioned media of these cells. Cell-associated PSCA migrates higher than secreted or shed PSCA on a 15% polyacrylamide gel. C: FACS analysis of mock-transfected 293T cells, PSCA-transfected 293T cells and LAPC-4 prostate cancer xenograft cells using an affinity purified polyclonal anti-PSCA antibody. Cells were not permeabilized in order to detect only surface expression. The y axis represents relative cell number and the x axis represents fluorescent staining intensity on a logarithmic scale.

FIG. 13. In situ hybridization of biotin-labeled PSCA probes to human metaphase cells from phytohemagglutinin-stimulated peripheral blood lymphocytes. The chromosome 8 homologues are identified with arrows; specific labeling was observed at 8q24.2. The inset shows partial

karyotypes of two chromosome 8 homologues illustrating specific labeling at 8q24.2 (arrowheads). Images were obtained using a Zeiss Axiophot microscope coupled to a cooled charge coupled device (CCD) camera. Separate images of DAPI stained chromosomes and the hybridization signal were merged using image analysis software (NU200 and Image 1.57).

5

FIG. 14. Flow Cytometric analysis of cell surface PSCA protein expression on prostate cancer xenograft (LAPC-9), prostate cancer cell line (LAPC-4) and normal prostate epithelial cells (PreC) using anti-PSCA monoclonal antibodies 1G8 (green) and 3E6 (red), mouse anti-PSCA polyclonal serum (blue), or control secondary antibody (black). See Example 5 for details.

10

FIG. 15. (a) An epitope map for each of the seven disclosed antibodies. (b) Epitope mapping of anti-PSCA monoclonal antibodies conducted by Western blot analysis of GST-PSCA fusion proteins.

15 FIG. 16. A schematic diagram showing that PSCA is a GPI-anchored protein.

FIG. 17. A photograph showing a FISH analysis of PSCA and c-myc Gene Copy No. in prostate cancer.

20 FIG. 18. A photograph showing FITC labeled 1G8 antibodies strongly bind PSCA protein on PSCA transfected LNCAP cells.

FIG. 19. A photograph showing FITC labeled 1G8 antibodies weakly bind PreC cells.

25 FIG. 20. A photograph showing in situ RNA hybridization of PSCA in normal prostate basal cells.

FIG. 21. PSCA immunostaining in primary prostate cancers. Representative paraffin-embedded sections from four patients were stained with anti-PSCA mAbs. The specimen from patient 1 demonstrates overexpression of PSCA protein in a Gleason grade 4 tumor (arrow) and

30

undetectable expression of PSCA in adjacent normal glands (arrowhead) using PSCA mAb 1G8. The positively staining cancer completely surrounds the normal glands. The specimen from patient 2 demonstrates heterogeneous staining in a Gleason grade 3 + 3/4 cancer. The Gleason pattern 3 glands (arrowhead) stain weakly compared with the larger, more cribriform appearing Gleason pattern 3/4 glands (arrow). The specimen from patient 3 demonstrates strong expression of PSCA by a poorly differentiated Gleason 5 (arrow) tumor with mAb 1G8. Patient 4 is a biopsy specimen showing no PSCA staining in the majority of a poorly differentiated tumor (arrowhead) and extremely weak staining in a cribriform focus identified in the specimen. The matched bone metastasis from patient 4 is shown in Figure 28.

10

FIG. 22. A photograph of a bone sample showing bone metastases of prostate cancer as determined by biotinylated 1G8 monoclonal antibody linked to horseradish peroxidase-conjugated streptavidin.

15

FIG. 23. A photograph of a bone sample showing bone metastases of prostate cancer as determined by biotinylated 1G8 monoclonal antibody linked to horseradish peroxidase-conjugated streptavidin.

20

FIG. 24. A photograph of a bone sample showing bone metastases of prostate cancer as determined by biotinylated 3E6 monoclonal antibody linked to horseradish peroxidase-conjugated streptavidin.

25

FIG. 25. A northern blot showing increased level of PSCA RNA in LAPC9, and transitional cell carcinoma of an advanced bladder carcinoma

30

FIG. 26. A photograph of a tissue undergoing early stage prostate cancer as determined by biotinylated 3E6 monoclonal antibody linked to horseradish peroxidase-conjugated streptavidin.

FIG. 27. A photograph of a bone sample showing bone metastases of prostate cancer as determined by hematoxylin stained 3E6 monoclonal antibody.

FIG. 28. PSCA immunostaining in prostate cancer bone metastases. The top panel shows the hematoxylin and eosin (left) and PSCA (right) staining of a bony lesion from patient 5. A single focus suspicious for cancer (arrow) was identified in the H and E section and confirmed by intense staining with anti-PSCA mAb 1G8 (arrow). The bottom panel shows the H and E (left) and PSCA staining of a bone lesion from patient 4. The primary lesion from patient 4 is depicted in Figure 21. Both the H and E and PSCA stains show diffuse bony involvement by prostate cancer (arrows). Again, PSCA immunostaining in the bone metastasis is uniform and intense.

10 FIG. 29. A photograph of a tissue undergoing early stage prostate cancer as determined by biotinylated 1G8 monoclonal antibody linked to horseradish peroxidase-conjugated streptavidin.

FIG. 30. A photograph showing that 1G8 binds LAPC9 cells as determined by hematoxylin staining.

15

FIG. 31. A photograph showing that 1G8 binds PSCA-transfected LnCaP cells.

FIG. 32. A photograph showing that 1G8 does not bind LnCaP cells (not transfected with PSCA).

20 FIG. 33. Flow cytometric recognition of PSCA on the cell surface of nonpermeabilized LAPC-9 human prostate cancer cells using mAbs 1G8, 2H9, 3E6, 3C5 and 4A10. Staining was compared to an irrelevant isotype control antibody.

FIG. 34. A photograph showing 293T cells transiently transfected with PSCA and immunoblotted with PSCA monoclonal antibodies. Monoclonal antibodies 2H9 and 3E6 binds deglycosylated PSCA but does not bind glycosylated PSCA in 293T cells. In contrast, monoclonal antibodies 1G8, 3C5, and 4A10 recognizes glycosylated PSCA.

FIG. 35. Immunofluorescent analysis demonstrating cell surface expression of PSCA in nonpermeabilized prostate cancer cells. LNCaP cells were stably transfected with PSCA and

30

stained with mAbs 1G8, 3E6, 3C5 and 4A10. Negative controls included irrelevant isotype antibody and LNCaP cells transfected with control vector, all of which showed no staining even after prolonged exposures.

5 FIG. 36. A photograph showing monoclonal antibody 2H9 binds LAPC9 cells.

Figure 37. A photograph showing immunological reactivity of anti-PSCA mAbs. (A) Immunoprecipitation of PSCA from 293T cells transiently transfected with PSCA using mAbs 1G8, 2H9, 3C5, 3E6 and 4A10. The control was an irrelevant murine IgG mAb. (B) Immunoblot
10 analysis of 293T cells transiently transfected with PSCA using the five anti-PSCA mAbs. mAbs 1G8, 3C5 and 4A10 all recognize equivalent molecular forms of PSCA, whereas mAbs 2H9 and 3E6 only weakly recognize deglycosylated forms of PSCA in 293T-PSCA cells in this assay.

Figure 38. Immunohistochemical staining of normal prostate with anti-PSCA mAbs. Examples
15 shown include a normal gland stained with an irrelevant isotype antibody as a negative control (arrow), PSCA mAb 3E6 and mAb 1G8. PSCA mAb 3E6 preferentially stains basal cells (arrow) when compared with secretory cells (arrowhead), whereas mAb 1G8 stains both basal (arrow) and secretory (arrowhead) cells equally. Also shown is strong staining of an atrophic single-layered gland from a normal prostate specimen stained with PSCA mAb 2H9.

20

Figure 39. Expression of PSCA protein in normal tissues. (A) Panel *a* shows staining of bladder transitional epithelium with mAb 1G8. Panel *b* shows colonic neuroendocrine cell staining with mAb 1G8. Double staining with chromogranin confirmed that the positive cells are of neuroendocrine origin (not shown). Panel *c* shows staining of collecting ducts (arrow) and tubules
25 with mAb 3E6. Panel *d* show staining of placental trophoblasts with mAb 3E6. (B) Northern blot analysis of PSCA mRNA expression. Total RNA from normal prostate, kidney, bladder and the LAPC-9 prostate cancer xenograft was analyzed using a PSCA specific probe (top panel). The same membrane was probed with actin to control of loading differences (bottom panel).

Figure 40. Targeting of mouse PSCA gene. (A) Panel *a* is a schematic drawing showing a strategy for creating a PSCA targeting vector. (B) Panel *b* is a photograph of a southern blot analysis of genomic DNA using 3' probe showing recovery of wild-type (+/+) and heterozygous (+/-) ES cells.

5

Figure 41. The upper panel is a schematic drawing of a strategy for generating transgenic mouse models of prostate cancer. The lower panel is a list of existing transgenic mouse models of prostate cancer.

10 Figure 42. A schematic drawing showing reporter gene constructs for transfection assay.

Figure 43. A bar graph showing the tissue-predominant expression (prostate and bladder cells) of the 9kb human PSCA upstream regulatory region having increased gene expression activity.

15 Figure 44. Bar graphs identifying prostate-predominant expression elements within PSCA upstream regions having increased gene expression activity, i.e., the 9kb, 6kb, 3kb, and 1kb PSCA regions.

20 Figure 45. A schematic drawing showing the design of transgenic vectors containing either a 9 kb or 6 kb human PSCA upstream region operatively linked to a detectable marker.

Figure 46. Photographs showing that the 9kb PSCA upstream region drives reporter gene expression in prostate, bladder and skin in vivo.

25 Figure 47. Photographs of multiple tissue northern blot analysis showing tissue specific expression patterns of human and murine PSCA RNA.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to Prostate Stem Cell Antigen (hereinafter "PSCA"). PSCA is a novel, glycosylphosphatidylinositol (GPI)-anchored cell surface antigen which is expressed in normal cells such prostate cells, urothelium, renal collecting ducts, colonic neuroendocrine cells, placenta, normal bladder and ureteral transitional epithelial cells (FIG. 16). PSCA, in addition to normal cells, is also overexpressed by both androgen-dependent and androgen-independent prostate cancer cells (FIG. 9-11), prostate cancer metastases to bone (FIG. 20-24 and 26-32), and bladder carcinomas (FIG. 6 and 25). The expression of PSCA in cancer, e.g., prostate cancer, appears to correlate with increasing grade. Further, overexpression of PSCA (i.e. higher expression than found in normal cells) in patients suffering from cancer, e.g., prostate cancer, appears to be indicative of poor prognosis.

PSCA mRNA is also expressed by a subset of basal cells in normal prostate. The basal cell epithelium is believed to contain the progenitor cells for the terminally differentiated secretory cells (Bonkhoff et al., 1994, Prostate 24: 114-118). Recent studies using cytokeratin markers suggest that the basal cell epithelium contains at least two distinct cellular subpopulations, one expressing cytokeratins 5 and 14 and the other cytokeratins 5, 8 and 18 (Bonkhoff and Remberger, 1996, Prostate 28: 98-106). The finding that PSCA is expressed by only a subset of basal cells suggests that PSCA may be a marker for one of these two basal cell lineages.

The biological function of PSCA is unknown. The Ly-6 gene family is involved in diverse cellular functions, including signal transduction and cell-cell adhesion. Signaling through SCA-2 has been demonstrated to prevent apoptosis in immature thymocytes (Noda et al., 1996, J. Exp. Med. 183: 2355-2360). Thy-1 is involved in T cell activation and transmits signals through src-like tyrosine kinases (Thomas et al., 1992, J. Biol. Chem. 267: 12317-12322). Ly-6 genes have been implicated both in tumorigenesis and in homotypic cell adhesion (Bamezai and Rock, 1995, Proc. Natl. Acad. Sci. USA 92: 4294-4298; Katz et al., 1994, Int. J. Cancer 59: 684-691; Brakenhoff et al., 1995, J. Cell Biol. 129: 1677-1689). Based on its restricted expression in basal cells and its homology to

Sca-2, we hypothesize that PSCA may play a role in stem/progenitor cell functions such as self-renewal (anti-apoptosis) and/or proliferation.

PSCA is highly conserved in mice and humans. The identification of a conserved gene which is predominantly restricted to prostate supports the hypothesis that PSCA may play an important role in normal prostate development.

In its various aspects, as described in detail below, the present invention provides PSCA proteins, antibodies, nucleic acid molecules, recombinant DNA molecules, transformed host cells, generation methods, assays, immunotherapeutic methods, transgenic animals, immunological and nucleic acid-based assays, and compositions.

PSCA PROTEINS

One aspect of the invention provides various PSCA proteins and peptide fragments thereof. As used herein, PSCA refers to a protein that has the amino acid sequence of human PSCA as provided in FIGS. 1B and 3, the amino acid sequence of the murine PSCA homologue as provided in FIG. 3, or the amino acid sequence of other mammalian PSCA homologues, as well as allelic variants and conservative substitution mutants of these proteins that have PSCA activity. The PSCA proteins of the invention include the specifically identified and characterized variants herein described, as well as allelic variants, conservative substitution variants and homologs that can be isolated/generated and characterized without undue experimentation following the methods outlined below. For the sake of convenience, all PSCA proteins will be collectively referred to as the PSCA proteins, the proteins of the invention, or PSCA.

The term "PSCA" includes all naturally occurring allelic variants, isoforms, and precursors of human PSCA as provided in FIGS. 1B and 3 and murine PSCA as provided in FIG. 3. In general, for example, naturally occurring allelic variants of human PSCA will share significant homology (e.g., 70 - 90%) to the PSCA amino acid sequence provided in FIGS. 1B and 3. Allelic variants, though possessing a slightly different amino acid sequence, may be expressed on the surface of prostate cells

as a GPI linked protein or may be secreted or shed. Typically, allelic variants of the PSCA protein will contain conservative amino acid substitutions from the PSCA sequence herein described or will contain a substitution of an amino acid from a corresponding position in a PSCA homologue such as, for example, the murine PSCA homologue described herein.

5

One class of PSCA allelic variants will be proteins that share a high degree of homology with at least a small region of the PSCA amino acid sequences presented in FIGS. 1B and 3, but will further contain a radical departure from the sequence, such as a non-conservative substitution, truncation, insertion or frame shift. Such alleles are termed mutant alleles of PSCA and represent proteins that typically do not perform the same biological functions.

10

Conservative amino acid substitutions can frequently be made in a protein without altering either the conformation or the function of the protein. Such changes include substituting any of isoleucine (I), valine (V), and leucine (L) for any other of these hydrophobic amino acids; aspartic acid (D) for glutamic acid (E) and vice versa; glutamine (Q) for asparagine (N) and vice versa; and serine (S) for threonine (T) and vice versa. Other substitutions can also be considered conservative, depending on the environment of the particular amino acid and its role in the three-dimensional structure of the protein. For example, glycine (G) and alanine (A) can frequently be interchangeable, as can alanine (A) and valine (V). Methionine (M), which is relatively hydrophobic, can frequently be interchanged with leucine and isoleucine, and sometimes with valine. Lysine (K) and arginine (R) are frequently interchangeable in locations in which the significant feature of the amino acid residue is its charge and the differing pK's of these two amino acid residues are not significant. Still other changes can be considered "conservative" in particular environments.

20

25

The amino acid sequence of human PSCA protein is provided in FIGS. 1B and 3. Human PSCA is comprised of a single subunit of 123 amino acids and contains an amino-terminal signal sequence, a carboxy-terminal GPI-anchoring sequence, and multiple N-glycosylation sites. PSCA shows 30% homology to stem cell antigen-2 (SCA-2), a member of the Thy-1/Ly-6 gene family, a group of cell surface proteins which mark the earliest phases of hematopoietic development. The amino acid

30

sequence of a murine PSCA homologue is shown in FIG. 3. Murine PSCA is a single subunit protein of 123 amino acids having approximately 70% homology to human PSCA and similar structural organization.

5 PSCA proteins may be embodied in many forms, preferably in isolated form. As used herein, a protein is said to be isolated when physical, mechanical or chemical methods are employed to remove the PSCA protein from cellular constituents that are normally associated with the protein. A skilled artisan can readily employ standard purification methods to obtain an isolated PSCA protein. A purified PSCA protein molecule will be substantially free of other proteins or molecules that
10 impair the binding of PSCA to antibody or other ligand. The nature and degree of isolation and purification will depend on the intended use. Embodiments of the PSCA protein include a purified PSCA protein and a functional, soluble PSCA protein. One example of a functional soluble PSCA protein has the amino acid sequence shown in FIG. 1B or a fragment thereof. In one form, such functional, soluble PSCA proteins or fragments thereof retain the ability to bind antibody or other
15 ligand.

The invention also provides peptides comprising biologically active fragments of the human and murine PSCA amino acid sequences shown in FIGS. 1B and 3. For example, the invention provides a peptide fragment having the amino acid sequence TARIRAVGLLTVISK, a peptide
20 fragment having the amino acid sequence VDDSQDYVVGKK, and SLNCVDDSQDYVVGK.

The peptides of the invention exhibit properties of PSCA, such as the ability to elicit the generation of antibodies that specifically bind an epitope associated with PSCA. Such peptide fragments of the PSCA proteins can be generated using standard peptide synthesis technology and
25 the amino acid sequences of the human or murine PSCA proteins disclosed herein. Alternatively, recombinant methods can be used to generate nucleic acid molecules that encode a fragment of the PSCA protein. In this regard, the PSCA-encoding nucleic acid molecules described herein provide means for generating defined fragments of PSCA.

As discussed below, peptide fragments of PSCA are particularly useful in: generating domain specific antibodies; identifying agents that bind to PSCA or a PSCA domain; identifying cellular factors that bind to PSCA or a PSCA domain; and isolating homologs or other allelic forms of human PSCA. PSCA peptides containing particularly interesting structures can be predicted and/or identified using various analytical techniques well known in the art, including, for example, the methods of Chou-Fasman, Garnier-Robson, Kyte-Doolittle, Eisenberg, Karplus-Schultz or Jameson-Wolf analysis, or on the basis of immunogenicity. As examples, hydrophobicity and Chou-Fasman plots of human PSCA are provided in FIGS. 4 and 5, respectively. Fragments containing such residues are particularly useful in generating subunit specific anti-PSCA antibodies or in identifying cellular factors that bind to PSCA.

The PSCA proteins of the invention may be useful for a variety of purposes, including but not limited to their use as diagnostic and/or prognostic markers of prostate cancer, the ability to elicit the generation of antibodies, and as targets for various therapeutic modalities, as further described below.

PSCA proteins may also be used to identify and isolate ligands and other agents that bind to PSCA. In the normal prostate, PSCA is expressed exclusively in a subset of basal cells, suggesting that PSCA may be used as a marker for a specific cell lineage within basal epithelium. In addition, applicants' results suggest that this set of basal cells represent the target of neoplastic transformation. Accordingly for example, therapeutic strategies designed to eliminate or modulate the molecular factors responsible for transformation may be specifically directed to this population of cells via the PSCA cell surface protein.

PSCA ANTIBODIES

The invention further provides antibodies (e.g., polyclonal, monoclonal, chimeric, and humanized antibodies) that bind to PSCA. The most preferred antibodies will selectively bind to PSCA and will not bind (or will bind weakly) to non-PSCA proteins. Anti-PSCA antibodies that are particularly contemplated include monoclonal and polyclonal antibodies as well as fragments thereof (e.g., recombinant proteins) containing the antigen binding domain and/or one or more complement

determining regions of these antibodies. These antibodies can be from any source, e.g., rat, dog, cat, pig, horse, mouse and human.

In one embodiment, the PSCA antibodies specifically bind to the extracellular domain of a PSCA protein, e.g., on the cell surface of prostate cancer cells from primary lesions and prostate cancer bone metastases. In other embodiments, the PSCA antibodies specifically bind to other domains of a PSCA protein or precursor. As will be understood by those skilled in the art, the regions or epitopes of a PSCA protein to which an antibody is directed may vary with the intended application. For example, antibodies intended for use in an immunoassay for the detection of membrane-bound PSCA on viable prostate cancer cells should be directed to an accessible epitope on membrane-bound PSCA. Examples of such antibodies are described the Examples which follow. Antibodies that recognize other epitopes may be useful for the identification of PSCA within damaged or dying cells, for the detection of secreted PSCA proteins or fragments thereof. The invention also encompasses antibody fragments that specifically recognize a PSCA protein. As used herein, an antibody fragment is defined as at least a portion of the variable region of the immunoglobulin molecule that binds to its target, i.e., the antigen binding region. Some of the constant region of the immunoglobulin may be included.

For example, the overexpression of PSCA in both androgen-dependent and androgen-independent prostate cancer cells, and the cell surface location of PSCA represent characteristics of an excellent marker for screening, diagnosis, prognosis, and follow-up assays and imaging methods. In addition, these characteristics indicate that PSCA may be an excellent target for therapeutic methods such as targeted antibody therapy, immunotherapy, and gene therapy.

PSCA antibodies of the invention may be particularly useful in diagnostic assays, imaging methodologies, and therapeutic methods in the management of prostate cancer. The invention provides various immunological assays useful for the detection of PSCA proteins and for the diagnosis of prostate cancer. Such assays generally comprise one or more PSCA antibodies capable of recognizing and binding a PSCA protein, and include various immunological assay formats well known in the art, including but not limited to various types of precipitation, agglutination,

complement fixation, radioimmunoassays (RIA), enzyme-linked immunosorbent assays (ELISA), enzyme-linked immunofluorescent assays (ELIFA) (H. Liu et al. Cancer Research 58: 4055-4060 (1998), immunohistochemical analysis and the like. In addition, immunological imaging methods capable of detecting prostate cancer are also provided by the invention, including but limited to
5 radioscintigraphic imaging methods using labeled PSCA antibodies. Such assays may be clinically useful in the detection, monitoring, and prognosis of prostate cancer.

In one embodiment, PSCA antibodies and fragments thereof (e.g., Fv, Fab', F(ab')₂) are used for detecting the presence of a prostate cancer, bladder carcinoma, bone metastases of prostate cancer,
10 PIN, or basal epithelial cell expressing a PSCA protein. The presence of such PSCA positive (+) cells within various biological samples, including serum, prostate and other tissue biopsy specimens, other tissues such as bone, urine, etc., may be detected with PSCA antibodies. In addition, PSCA antibodies may be used in various imaging methodologies, such as immunoscintigraphy with Indium-111 (or other isotope) conjugated antibody. For example, an
15 imaging protocol similar to the one recently described using an In-111 conjugated anti-PSMA antibody may be used to detect recurrent and metastatic prostate carcinomas (Sodee et al., 1997, Clin Nuc Med 21: 759-766). In relation to other markers of prostate cancer, such as PSMA for example, PSCA may be particularly useful for targeting androgen independent prostate cancer cells. PSCA antibodies may also be used therapeutically to inhibit PSCA function.

20 PSCA antibodies may also be used in methods for purifying PSCA proteins and peptides and for isolating PSCA homologues and related molecules. For example, in one embodiment, the method of purifying a PSCA protein comprises incubating a PSCA antibody, which has been coupled to a solid matrix, with a lysate or other solution containing PSCA under conditions which permit the PSCA
25 antibody to bind to PSCA; washing the solid matrix to eliminate impurities; and eluting the PSCA from the coupled antibody. Additionally, PSCA antibodies may be used to isolate PSCA positive cells using cell sorting and purification techniques. The presence of PSCA on prostate tumor cells (alone or in combination with other cell surface markers) may be used to distinguish and isolate human prostate cancer cells from other cells. In particular, PSCA antibodies may be used to
30 isolate prostate cancer cells from xenograft tumor tissue, from cells in culture, etc., using antibody-

based cell sorting or affinity purification techniques. Other uses of the PSCA antibodies of the invention include generating anti-idiotypic antibodies that mimic the PSCA protein, e.g., a monoclonal anti-idiotypic antibody reactive with an idiotype on any of the monoclonal antibodies of the invention such as 1G8, 2A2, 2H9, 3C5, 3E6, 3G3, and 4A10.

5

The ability to generate large quantities of relatively pure human PSCA positive prostate cancer cells which can be grown in tissue culture or as xenograft tumors in animal models (e.g., SCID or other immune deficient mice) provides many advantages, including, for example, permitting the evaluation of various transgenes or candidate therapeutic compounds on the growth or other phenotypic characteristics of a relatively homogeneous population of prostate cancer cells. Additionally, this feature of the invention also permits the isolation of highly enriched preparations of human PSCA positive prostate cancer specific nucleic acids in quantities sufficient for various molecular manipulations. For example, large quantities of such nucleic acid preparations will assist in the identification of rare genes with biological relevance to prostate cancer disease progression.

15

Another valuable application of this aspect of the invention is the ability to isolate, analyze and experiment with relatively pure preparations of viable PSCA positive prostate tumor cells cloned from individual patients with locally advanced or metastatic disease. In this way, for example, an individual patient's prostate cancer cells may be expanded from a limited biopsy sample and then tested for the presence of diagnostic and prognostic genes, proteins, chromosomal aberrations, gene expression profiles, or other relevant genotypic and phenotypic characteristics, without the potentially confounding variable of contaminating cells. In addition, such cells may be evaluated for neoplastic aggressiveness and metastatic potential in animal models. Similarly, patient-specific prostate cancer vaccines and cellular immunotherapeutics may be created from such cell preparations.

20

25

Various methods for the preparation of antibodies are well known in the art. For example, antibodies may be prepared by immunizing a suitable mammalian host using a PSCA protein, peptide, or fragment, in isolated or immunoconjugated form (Harlow, Antibodies, Cold Spring Harbor Press,

30

NY (1989)). In addition, fusion proteins of PSCA may also be used, such as a PSCA GST-fusion protein. Cells expressing or overexpressing PSCA may also be used for immunizations. Similarly, any cell engineered to express PSCA may be used. This strategy may result in the production of monoclonal antibodies with enhanced capacities for recognizing endogenous PSCA. For example, using standard technologies described in Example 5 and standard hybridoma protocols (Harlow and Lane, 1988, Antibodies: A Laboratory Manual. (Cold Spring Harbor Press)), hybridomas producing monoclonal antibodies designated 1G8 (ATCC No. HB-12612), 2A2 (ATCC No. HB-12613), 2H9 (ATCC No. HB-12614), 3C5 (ATCC No. HB-12616), 3E6 (ATCC No. HB-12618), and 3G3 (ATCC No. HB-12615), 4A10 (ATCC No. HB-12617) were generated. These antibody were deposited on December 11, 1998 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852.

Chimeric antibodies of the invention are immunoglobulin molecules that comprise a human and non-human portion. The antigen combining region (variable region) of a chimeric antibody can be derived from a non-human source (e.g. murine) and the constant region of the chimeric antibody which confers biological effector function to the immunoglobulin can be derived from a human source. The chimeric antibody should have the antigen binding specificity of the non-human antibody molecule and the effector function conferred by the human antibody molecule.

- In general, the procedures used to produce chimeric antibodies can involve the following steps:
- a) identifying and cloning the correct gene segment encoding the antigen binding portion of the antibody molecule; this gene segment (known as the VDJ, variable, diversity and joining regions for heavy chains or VJ, variable, joining regions for light chains or simply as the V or variable region) may be in either the cDNA or genomic form;
 - b) cloning the gene segments encoding the constant region or desired part thereof;
 - c) ligating the variable region with the constant region so that the complete chimeric antibody is encoded in a form that can be transcribed and translated;
 - d) ligating this construct into a vector containing a selectable marker and gene control regions such as promoters, enhancers and poly(A) addition signals;
 - e) amplifying this construct in bacteria;

- f) introducing this DNA into eukaryotic cells (transfection) most often mammalian lymphocytes;
- g) selecting for cells expressing the selectable marker;
- h) screening for cells expressing the desired chimeric antibody; and
- k) testing the antibody for appropriate binding specificity and effector functions.

5

Antibodies of several distinct antigen binding specificities have been manipulated by these protocols to produce chimeric proteins [e.g. anti-TNP: Boulianne et al., Nature 312:643 (1984); and anti-tumor antigens: Sahagan et al., J. Immunol. 137:1066 (1986)]. Likewise, several different effector functions have been achieved by linking new sequences to those encoding the antigen binding region. Some of these include enzymes [Neuberger et al., Nature 312:604 (1984)], immunoglobulin constant regions from another species and constant regions of another immunoglobulin chain [Sharon et al., Nature 309:364 (1984); Tan et al., J. Immunol. 135:3565-3567 (1985)]. Additionally, procedures for modifying antibody molecules and for producing chimeric antibody molecules using homologous recombination to target gene modification have been described (Fell et al., Proc. Natl. Acad. Sci. USA 86:8507-8511 (1989)).

10

15

These antibodies are capable of binding to PSCA, e.g., on the cell surface of prostate cancer cells, thereby confirming the cell surface localization of PSCA. Because these mAbs recognize epitopes on the exterior of the cell surface, they have utility for prostate cancer diagnosis and therapy. For example, these mAbs were used to locate sites of metastatic disease (Example 6). Another possibility is that they may be used (e.g., systemically) to target prostate cancer cells therapeutically when used alone or conjugated to a radioisotope or other toxin.

20

PSCA mAbs stain the cell surface in a punctate manner (see Example 5), suggesting that PSCA may be localized to specific regions of the cell surface. GPI-anchored proteins are known to cluster in detergent-insoluble glycolipid-enriched microdomains (DIGS) of the cell surface. These microdomains, which include caveolae and shingolipid-cholesterol rafts, are believed to play critical roles in signal transduction and molecular transport. Thy-1, a homologue of PSCA, has previously been shown to transmit signals to src kinases through interaction in lipid-microdomains. Subcellular fractionation experiments in our laboratory confirm the presence of PSCA in DIGS.

25

30

Additionally, some of the antibodies of the invention are internalizing antibodies, i.e., the antibodies are internalized into the cell upon or after binding. Further, some of the antibodies induce inhibition of PSCA positive cancer cell growth.

5

A characterization of these antibodies, e.g., in prostate cancer specimens, demonstrates that PSCA protein is overexpressed in prostate cancers relative to normal cells and its expression appears to be up-regulated during prostate cancer progression and metastasis. These antibodies are useful in studies of PSCA biology and function, as well as in vivo targeting of PSCA associated cancers, e.g., human prostate cancer, prostate cancer metastases to bone, and bladder carcinomas.

10

The amino acid sequence of PSCA presented herein may be used to select specific regions of the PSCA protein for generating antibodies. For example, hydrophobicity and hydrophilicity analyses of the PSCA amino acid sequence may be used to identify hydrophilic regions in the PSCA structure.

15

Regions of the PSCA protein that show immunogenic structure, as well as other regions and domains, can readily be identified using various other methods known in the art, such as Chou-Fasman, Garnier-Robson, Kyte-Doolittle, Eisenberg, Karplus-Schultz or Jameson-Wolf analysis. Fragments containing these residues are particularly suited in generating specific classes of anti-PSCA antibodies. Particularly useful fragments include, but are not limited to, the sequences

20

TARIRAVGLLTVISK and SLNCVDDSQDYVVGK.

As described in Example 2, below, a rabbit polyclonal antibody was generated against the former fragment, prepared as a synthetic peptide, and affinity purified using a PSCA-glutathione S transferase fusion protein. Recognition of PSCA by this antibody was established by immunoblot and immunoprecipitation analysis of extracts of 293T cells transfected with PSCA and a GST-PSCA fusion protein. This antibody also identified the cell surface expression of PSCA in PSCA-transfected 293T and LAPC-4 cells using fluorescence activated cell sorting (FACS).

25

Additionally, a sheep polyclonal antibody was generated against the latter fragment, prepared as a synthetic peptide, and affinity purified using a peptide Affi-gel column (also by the method of

30

Example 2). Recognition of PSCA by this antibody was established by immunoblot and immunoprecipitation analysis of extracts of LNCaP cells transfected with PSCA. This antibody also identified the cell surface expression of PSCA in PSCA-transfected LNCaP cells using fluorescence activated cell sorting (FACS) and immunohistochemistry analysis.

5

Methods for preparing a protein for use as an immunogen and for preparing immunogenic conjugates of a protein with a carrier such as BSA, KLH, or other carrier proteins are well known in the art. In some circumstances, direct conjugation using, for example, carbodiimide reagents may be used; in other instances linking reagents such as those supplied by Pierce Chemical Co., Rockford, IL, may be effective. Administration of a PSCA immunogen is conducted generally by injection over a suitable time period and with use of a suitable adjuvant, as is generally understood in the art. During the immunization schedule, titers of antibodies can be taken to determine adequacy of antibody formation.

15 While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, monoclonal antibody preparations are preferred. Immortalized cell lines which secrete a desired monoclonal antibody may be prepared using the standard method of Kohler and Milstein or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known. The immortalized cell lines secreting the desired antibodies are screened by immunoassay in which the antigen is the PSCA protein or PSCA fragment. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production in ascites fluid.

25 The desired monoclonal antibodies are then recovered from the culture supernatant or from the ascites supernatant. Fragments of the monoclonal antibodies of the invention or the polyclonal antisera (e.g., Fab, F(ab')₂, Fv fragments, fusion proteins) which contain the immunologically significant portion (i.e., a portion that recognizes and binds PSCA) can be used as antagonists, as well as the intact antibodies. Humanized antibodies directed against PSCA is also useful. As used herein, a humanized PSCA antibody is an immunoglobulin molecule which is capable of binding to PSCA and which comprises a FR region having substantially the amino acid sequence of a human

30

immunoglobulin and a CDR having substantially the amino acid sequence of non-human immunoglobulin or a sequence engineered to bind PSCA.

5 Use of immunologically reactive fragments, such as the Fab, Fab', or F(ab')₂ fragments is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin. The invention also provides pharmaceutical compositions having the monoclonal antibodies or anti-idiotypic monoclonal antibodies of the invention.

10 The generation of monoclonal antibodies (MAbs) capable of binding to cell surface PSCA are described in Example 5. Epitope mapping of these MAbs indicates that they recognize different epitopes on the PSCA protein. For example, one recognizes an epitope within the carboxy-terminal region and the other recognizing an epitope within the amino-terminal region. Such PSCA antibodies may be particularly well suited to use in a sandwich-formatted ELISA, given their differing epitope binding characteristics.

15 The antibodies or fragments may also be produced, using current technology, by recombinant means. Regions that bind specifically to the desired regions of the PSCA protein can also be produced in the context of chimeric or CDR grafted antibodies of multiple species origin. The invention includes a monoclonal antibody, the antigen-binding region of which competitively inhibits the
20 immunospecific binding of any of the monoclonal antibodies of the invention to its target antigen. Further, the invention provides recombinant proteins comprising the antigen-binding region of any the monoclonal antibodies of the invention.

25 The antibody or fragment thereof of the invention may be labeled with a detectable marker or conjugated to a second molecule, such as a therapeutic agent (e.g., a cytotoxic agent) thereby resulting in an immunoconjugate. For example, the therapeutic agent includes, but is not limited to, an anti-tumor drug, a toxin, a radioactive agent, a cytokine, a second antibody or an enzyme. Further, the invention provides an embodiment wherein the antibody of the invention is linked to an enzyme that converts a prodrug into a cytotoxic drug.

The immunoconjugate can be used for targeting the second molecule to a PSCA positive cell (Vitetta, E.S. et al., 1993, Immunotoxin therapy, in DeVita, Jr., V.T. et al., eds, Cancer: Principles and Practice of Oncology, 4th ed., J.B. Lippincott Co., Philadelphia, 2624-2636).

5

Examples of cytotoxic agents include, but are not limited to ricin, doxorubicin, daunorubicin, taxol, ethidum bromide, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicine, dihydroxy anthracin dione, actinomycin D, diphtheria toxin, *Pseudomonas* exotoxin (PE) A, PE40, abrin, and glucocorticoid and other chemotherapeutic agents, as well as radioisotopes. Suitable
10 detectable markers include, but are not limited to, a radioisotope, a fluorescent compound, a bioluminescent compound, chemiluminescent compound, a metal chelator or an enzyme.

Additionally, the recombinant protein of the invention comprising the antigen-binding region of any of the monoclonal antibodies of the invention can be used to treat cancer. In such a
15 situation, the antigen-binding region of the recombinant protein is joined to at least a functionally active portion of a second protein having therapeutic activity. The second protein can include, but is not limited to, an enzyme, lymphokine, oncostatin or toxin. Suitable toxins include doxorubicin, daunorubicin, taxol, ethidum bromide, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicine, dihydroxy anthracin dione, actinomycin D, diphtheria toxin,
20 *Pseudomonas* exotoxin (PE) A, PE40, ricin, abrin, glucocorticoid and radioisotopes.

Techniques for conjugating or joining therapeutic agents to antibodies are well known (see, e.g., Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc.
25 1985); Hellstrom et al., "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera et al. (eds.), pp. 475-506 (1985); and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev., 62:119-58 (1982)). The

use of PSCA antibodies as therapeutic agents is further described in the subsection "PROSTATE CANCER IMMUNOTHERAPY" below.

PSCA-ENCODING NUCLEIC ACID MOLECULES

Another aspect of the invention provides various nucleic acid molecules encoding PSCA proteins and fragments thereof, preferably in isolated form, including DNA, RNA, DNA/RNA hybrid, and related molecules, nucleic acid molecules complementary to the PSCA coding sequence or a part thereof, and those which hybridize to the PSCA gene or to PSCA-encoding nucleic acids.

Particularly preferred nucleic acid molecules will have a nucleotide sequence substantially identical to or complementary to the human or murine DNA sequences herein disclosed. Specifically contemplated are genomic DNA, cDNAs, ribozymes, and antisense molecules, as well as nucleic acids based on an alternative backbone or including alternative bases, whether derived from natural sources or synthesized.

For example, antisense molecules can be RNAs or other molecules, including peptide nucleic acids (PNAs) or non-nucleic acid molecules such as phosphorothioate derivatives, that specifically bind DNA or RNA in a base pair-dependent manner. A skilled artisan can readily obtain these classes of nucleic acid molecules using the herein described *PSCA* sequences. For convenience, PSCA-encoding nucleic acid molecules will be referred to herein as PSCA-encoding nucleic acid molecules, PSCA genes, or PSCA sequences.

The nucleotide sequence of a cDNA encoding one allelic form of human PSCA is provided in FIG. 1A. The nucleotide sequence of a cDNA encoding a murine PSCA homologue ("murine PSCA") is provided in FIG. 2. Genomic clones of human and murine PSCA have also been isolated, as described in Example 4. Both the human and murine genomic clones contain three exons encoding the translated and 3' untranslated regions of the PSCA gene. A fourth exon encoding a 5' untranslated region is presumed to exist based on PSCA's homology to other members of the Ly-6 and Thy-1 gene families (FIG. 8).

The human PSCA gene maps to chromosome 8q24.2. Human stem cell antigen 2 (RIG-E), as well as one other recently identified human Ly-6 homologue (E48) are also localized to this region, suggesting that a large family of related genes may exist at this locus (Brakenhoff et al., 1995, supra; Mao et al., 1996, Proc. Natl. Acad. Sci. USA 93: 5910-5914). Intriguingly, chromosome 8q
5 has been reported to be a region of allelic gain and amplification in a majority of advanced and recurrent prostate cancers (Cher et al., 1994, Genes Chrom. Cancer 11: 153-162). c-myc localizes proximal to PSCA at chromosome 8q24 and extra copies of c-myc (either through allelic gain or amplification) have been found in 68% of primary prostate tumors and 96% of metastases (Jenkins et al., 1997, Cancer Res. 57: 524-531).

10

Embodiments of the PSCA-encoding nucleic acid molecules of the invention include primers, which allow the specific amplification of nucleic acid molecules of the invention or of any specific parts thereof, and probes that selectively or specifically hybridize to nucleic acid molecules of the invention or to any part thereof. The nucleic acid probes can be labeled with a detectable marker.

15

Examples of a detectable marker include, but are not limited to, a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator or an enzyme. Such labeled probes can be used to diagnosis the presence of a PSCA protein as a means for diagnosing cell expressing a PSCA protein. Technologies for generating DNA and RNA probes are well known.

20

As used herein, a nucleic acid molecule is said to be "isolated" when the nucleic acid molecule is substantially separated from contaminant nucleic acid molecules that encode polypeptides other than PSCA. A skilled artisan can readily employ nucleic acid isolation procedures to obtain an isolated PSCA-encoding nucleic acid molecule.

25

The invention further provides fragments of the PSCA-encoding nucleic acid molecules of the present invention. As used herein, a fragment of a PSCA-encoding nucleic acid molecule refers to a small portion of the entire PSCA-encoding sequence. The size of the fragment will be determined by its intended use.

30

For example, if the fragment is chosen so as to encode an active portion of the PSCA protein, such an active domain, effector binding site or GPI binding domain, then the fragment will need to be large enough to encode the functional region(s) of the PSCA protein. If the fragment is to be used as a nucleic acid probe or PCR primer, then the fragment length is chosen so as to obtain a relatively small number of false positives during probing/priming.

Fragments of human *PSCA* that are particularly useful as selective hybridization probes or PCR primers can be readily identified from the entire *PSCA* sequence using art-known methods. One set of PCR primers that are useful for RT-PCR analysis comprise 5' - TGCTTGCCCTGTTGATGGCAG - and 3' - CCAGAGCAGCAGGCCGAGTGCA -.

METHODS FOR ISOLATING OTHER PSCA-ENCODING NUCLEIC ACID MOLECULES

The PSCA-encoding nucleic acid molecules described herein enable the isolation of PSCA homologues, alternatively sliced isoforms, allelic variants, and mutant forms of the PSCA protein as well as their coding and gene sequences. The most preferred source of PSCA homologs are mammalian organisms.

For example, a portion of the PSCA-encoding sequence herein described can be synthesized and used as a probe to retrieve DNA encoding a member of the PSCA family of proteins from organisms other than human, allelic variants of the human PSCA protein herein described, and genomic sequence containing the *PSCA* gene. Oligomers containing approximately 18-20 nucleotides (encoding about a 6-7 amino acid stretch) are prepared and used to screen genomic DNA or cDNA libraries to obtain hybridization under stringent conditions or conditions of sufficient stringency to eliminate an undue level of false positives. In a particular embodiment, cDNA encoding human PSCA was used to isolate a full length cDNA encoding the murine PSCA homologue as described in Example 3 herein. The murine clone encodes a protein with 70% amino acid identity to human PSCA.

In addition, the amino acid sequence of the human PSCA protein may be used to generate antibody probes to screen expression libraries prepared from cells. Typically, polyclonal antiserum from mammals such as rabbits immunized with the purified protein (as described below) or monoclonal antibodies can be used to probe an expression library, prepared from a target organism, to obtain the appropriate coding sequence for a PSCA homologue. The cloned cDNA sequence can be expressed as a fusion protein, expressed directly using its own control sequences, or expressed by constructing an expression cassette using control sequences appropriate to the particular host used for expression of the enzyme.

- Genomic clones containing PSCA genes may be obtained using molecular cloning methods well known in the art. In one embodiment, a human genomic clone of approximately 14kb containing exons 1-4 of the PSCA gene was obtained by screening a lambda phage library with a human PSCA cDNA probe, as more completely described in Example 4 herein. In another embodiment, a genomic clone of approximately 10kb containing the murine PSCA gene was obtained by screening a murine BAC (bacterial artificial chromosome) library with a murine PSCA cDNA (also described in Example 4).

Additionally, pairs of oligonucleotide primers can be prepared for use in a polymerase chain reaction (PCR) to selectively amplify/clone a PSCA-encoding nucleic acid molecule, or fragment thereof. A PCR denature/anneal/extend cycle for using such PCR primers is well known in the art and can readily be adapted for use in isolating other PSCA-encoding nucleic acid molecules. Regions of the human PSCA gene that are particularly well suited for use as a probe or as primers can be readily identified.

Non-human homologues of *PSCA*, naturally occurring allelic variants of *PSCA* and genomic *PSCA* sequences will share a high degree of homology to the human *PSCA* sequences herein described. In general, such nucleic acid molecules will hybridize to the human *PSCA* sequence under stringent conditions. Such sequences will typically contain at least 70% homology, preferably at least 80%, most preferably at least 90% homology to the human *PSCA* sequence.

Stringent conditions are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015M NaCl/0.0015M sodium nitrate/0.1% SDS at 50°C., or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C.

Another example is use of 50% formamide, 5 x SSC (0.75M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C., with washes at 42°C. in 0.2 x SSC and 0.1% SDS. A skilled artisan can readily determine and vary the stringency conditions appropriately to obtain a clear and detectable hybridization signal.

RECOMBINANT DNA MOLECULES CONTAINING PSCA-ENCODING NUCLEIC ACIDS

Also provided are recombinant DNA molecules (rDNAs) that contain a PSCA-encoding sequences as herein described, or a fragment thereof. As used herein, a rDNA molecule is a DNA molecule that has been subjected to molecular manipulation *in vitro*. Methods for generating rDNA molecules are well known in the art, for example, see Sambrook *et al.*, *Molecular Cloning* (1989). In the preferred rDNA molecules of the present invention, a PSCA-encoding DNA sequence that encodes a PSCA protein or a fragment of PSCA, is operably linked to one or more expression control sequences and/or vector sequences. The rDNA molecule can encode either the entire PSCA protein, or can encode a fragment of the PSCA protein.

The choice of vector and/or expression control sequences to which the PSCA-encoding sequence is operably linked depends directly, as is well known in the art, on the functional properties desired, e.g., protein expression, and the host cell to be transformed. A vector contemplated by the present invention is at least capable of directing the replication or insertion into the host chromosome, and preferably also expression, of the PSCA-encoding sequence included in the rDNA molecule.

Expression control elements that are used for regulating the expression of an operably linked protein encoding sequence are known in the art and include, but are not limited to, inducible promoters, constitutive promoters, secretion signals, enhancers, transcription terminators and other regulatory elements. Preferably, an inducible promoter that is readily controlled, such as being responsive to a nutrient in the host cell's medium, is used.

In one embodiment, the vector containing a PSCA-encoding nucleic acid molecule will include a prokaryotic replicon, i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule intrachromosomally in a prokaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, vectors that include a prokaryotic replicon may also include a gene whose expression confers a detectable marker such as a drug resistance. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline.

Vectors that include a prokaryotic replicon can further include a prokaryotic or viral promoter capable of directing the expression (transcription and translation) of the PSCA-encoding sequence in a bacterial host cell, such as *E. coli*. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention. Various viral vectors well known to those skilled in the art may also be used, such as, for example, a number of well known retroviral vectors.

Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can also be used to variant rDNA molecules that contain a PSCA-encoding sequence. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are PSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), the vector pCDM8 described herein, and the like eukaryotic expression vectors.

Eukaryotic cell expression vectors used to construct the rDNA molecules of the present invention may further include a selectable marker that is effective in an eukaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance marker is the gene whose expression results in neomycin resistance, i.e., the neomycin phosphotransferase (*neo*) gene. Southern *et al.*, *J Mol Anal Genet* (1982) 1:327-341. Alternatively, the selectable marker can be present on a separate plasmid, and the two vectors are introduced by cotransfection of the host cell, and selected by culturing in the presence of the appropriate drug for the selectable marker.

- 10 In accordance with the practice of the invention, the vector can be a plasmid, cosmid or phage vector encoding the cDNA molecule discussed above. Additionally, the invention provides a host-vector system comprising the plasmid, cosmid or phage vector transfected into a suitable eucaryotic host cell. Examples of suitable eucaryotic host cells include a yeast cell, a plant cell, or an animal cell, such as a mammalian cell. Examples of suitable cells include the LnCaP, LAPC-4, and other prostate cancer cell lines. The host-vector system is useful for the production of a PSCA protein. Alternatively, the host cell can be prokaryotic, such as a bacterial cell.

TRANSFORMED HOST CELLS

- 20 The invention further provides host cells transformed with a nucleic acid molecule that encodes a PSCA protein or a fragment thereof. The host cell can be either prokaryotic or eukaryotic. Eukaryotic cells useful for expression of a PSCA protein are not limited, so long as the cell line is compatible with cell culture methods and compatible with the propagation of the expression vector and expression of a PSCA gene. Preferred eukaryotic host cells include, but are not limited to, yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Prostate cancer cell lines, such as the LnCaP and LAPC-4 cell lines may also be used. Any prokaryotic host can be used to express a PSCA-encoding rDNA molecule. The preferred prokaryotic host is *E. coli*.

Transformation of appropriate cell hosts with an rDNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used and host system employed. With regard to transformation of prokaryotic host cells, electroporation and salt treatment methods are typically employed, see, for example, Cohen *et al.*, *Proc Acad Sci USA* (1972) 69:2110; and Maniatis *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). With regard to transformation of vertebrate cells with vectors containing rDNAs, electroporation, cationic lipid or salt treatment methods are typically employed, see, for example, Graham *et al.*, *Virol* (1973) 52:456; Wigler *et al.*, *Proc Natl Acad Sci USA* (1979) 76:1373-76.

Successfully transformed cells, i.e., cells that contain an rDNA molecule of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the rDNA using a method such as that described by Southern, *J Mol Biol* (1975) 98:503, or Berent *et al.*, *Biotech* (1985) 3:208 or the proteins produced from the cell assayed via an immunological method.

RECOMBINANT METHODS OF GENERATING PSCA PROTEINS

The invention further provides methods for producing a PSCA protein using one of the PSCA-encoding nucleic acid molecules herein described. In general terms, the production of a recombinant PSCA protein typically can involve the following steps (Maniatis, *supra*).

First, a nucleic acid molecule is obtained that encodes a PSCA protein or a fragment thereof, such as the nucleic acid molecule depicted in FIG. 1A. The PSCA-encoding nucleic acid molecule is then preferably placed in an operable linkage with suitable control sequences, as described above, to generate an expression unit containing the PSCA-encoding sequence. The expression unit is used to transform a suitable host and the transformed host is cultured under conditions that allow the production of the PSCA protein. Optionally the PSCA protein is isolated from the medium or from

the cells; recovery and purification of the protein may not be necessary in some instances where some impurities may be tolerated.

Each of the foregoing steps may be done in a variety of ways. For example, the desired coding sequences may be obtained from genomic fragments and used directly in an appropriate host. The construction of expression vectors that are operable in a variety of hosts is accomplished using an appropriate combination of replicons and control sequences. The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene and were discussed in detail earlier. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to provide an excisable gene to insert into these vectors. A skilled artisan can readily adapt any host/expression system known in the art for use with PSCA-encoding sequences to produce a PSCA protein.

ASSAYS FOR IDENTIFYING PSCA LIGANDS AND OTHER BINDING AGENTS

Another aspect of the invention relates to assays and methods that can be used to detect and identify PSCA ligands and other agents and cellular constituents that bind to PSCA. Specifically, PSCA ligands and other agents and cellular constituents that bind to PSCA can be identified by the ability of the PSCA ligand or other agent or constituent to bind to PSCA and/or the ability to inhibit/stimulate PSCA activity. Assays for PSCA activity (e.g., binding) using a PSCA protein are suitable for use in high through-put screening methods.

In one embodiment, the assay comprises mixing PSCA with a test agent or cellular extract. After mixing under conditions that allow association of PSCA with the agent or component of the extract, the mixture is analyzed to determine if the agent/component is bound to PSCA. Binding agents/components are identified as being able to bind to PSCA. Alternatively or consecutively, PSCA activity can be directly assessed as a means for identifying agonists and antagonists of PSCA activity.

Alternatively, targets that bind to a PSCA protein can be identified using a yeast two-hybrid system (Fields, S. and Song, O. (1989), Nature 340:245-246) or using a binding-capture assay (Harlow, supra). In the yeast two hybrid system, an expression unit encoding a fusion protein made up of one subunit of a two subunit transcription factor and the PSCA protein is introduced and expressed in a yeast cell. The cell is further modified to contain (1) an expression unit encoding a detectable marker whose expression requires the two subunit transcription factor for expression and (2) an expression unit that encodes a fusion protein made up of the second subunit of the transcription factor and a cloned segment of DNA. If the cloned segment of DNA encodes a protein that binds to the PSCA protein, the expression results in the interaction of the PSCA and the encoded protein. This brings the two subunits of the transcription factor into binding proximity, allowing reconstitution of the transcription factor. This results in the expression of the detectable marker. The yeast two hybrid system is particularly useful in screening a library of cDNA encoding segments for cellular binding partners of PSCA.

PSCA proteins which may be used in the above assays include, but are not limited to, an isolated PSCA protein, a fragment of a PSCA protein, a cell that has been altered to express a PSCA protein, or a fraction of a cell that has been altered to express a PSCA protein. Further, the PSCA protein can be the entire PSCA protein or a defined fragment of the PSCA protein. It will be apparent to one of ordinary skill in the art that so long as the PSCA protein can be assayed for agent binding, e.g., by a shift in molecular weight or activity, the present assay can be used.

The method used to identify whether an agent/cellular component binds to a PSCA protein will be based primarily on the nature of the PSCA protein used. For example, a gel retardation assay can be used to determine whether an agent binds to PSCA or a fragment thereof. Alternatively, immunodetection and biochip technologies can be adopted for use with the PSCA protein. A skilled artisan can readily employ numerous art-known techniques for determining whether a particular agent binds to a PSCA protein.

Agents and cellular components can be further tested for the ability to modulate the activity of a PSCA protein using a cell-free assay system or a cellular assay system. As the activities of the PSCA protein become more defined, functional assays based on the identified activity can be employed.

5 As used herein, an agent is said to antagonize PSCA activity when the agent reduces PSCA activity. The preferred antagonist will selectively antagonize PSCA, not affecting any other cellular proteins. Further, the preferred antagonist will reduce PSCA activity by more than 50%, more preferably by more than 90%, most preferably eliminating all PSCA activity.

10 As used herein, an agent is said to agonize PSCA activity when the agent increases PSCA activity. The preferred agonist will selectively agonize PSCA, not affecting any other cellular proteins. Further, the preferred antagonist will increase PSCA activity by more than 50%, more preferably by more than 90%, most preferably more than doubling PSCA activity.

15 Agents that are assayed in the above method can be randomly selected or rationally selected or designed. As used herein, an agent is said to be randomly selected when the agent is chosen randomly without considering the specific sequences of the PSCA protein. An example of randomly selected agents is the use of a chemical library or a peptide combinatorial library, or a growth broth of an organism or plant extract.

20 As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a nonrandom basis that takes into account the sequence of the target site and/or its conformation in connection with the agent's action. Agents can be rationally selected or rationally designed by utilizing the peptide sequences that make up the PSCA protein. For example, a rationally selected
25 peptide agent can be a peptide whose amino acid sequence is identical to a fragment of a PSCA protein.

The agents tested in the methods of the present invention can be, as examples, peptides, small molecules, and vitamin derivatives, as well as carbohydrates. A skilled artisan can readily recognize
30 that there is no limit as to the structural nature of the agents used in the present screening method.

One class of agents of the present invention are peptide agents whose amino acid sequences are chosen based on the amino acid sequence of the PSCA protein. Small peptide agents can serve as competitive inhibitors of PSCA protein assembly.

- 5 Peptide agents can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, the DNA encoding these peptides may be synthesized using commercially available oligonucleotide synthesis instrumentation and produced recombinantly using standard recombinant production systems. The production using solid phase peptide synthesis is necessitated if non-gene-encoded amino acids are to be included.

10

- Another class of agents of the present invention are antibodies immunoreactive with critical positions of the PSCA protein. As described above, antibodies are obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions, those portions of the PSCA protein intended to be targeted by the antibodies. Critical regions may include the domains identified in FIG. 15. Such agents can be used in competitive binding studies to identify second generation inhibitory agents.

- The cellular extracts tested in the methods of the present invention can be, as examples, aqueous extracts of cells or tissues, organic extracts of cells or tissues or partially purified cellular fractions.
- 20 A skilled artisan can readily recognize that there is no limit as to the source of the cellular extract used in the screening method of the present invention.

- Agents that bind a PSCA protein, such as a PSCA antibody, can be used to modulate the activity of PSCA, to target anticancer agents to appropriate mammalian cells, or to identify agents that block the interaction with PSCA. Cells expressing PSCA can be targeted or identified by using an agent that binds to PSCA.

- How the PSCA binding agents will be used depends on the nature of the PSCA binding agent. For example, a PSCA binding agent can be used to: deliver conjugated toxins, such a diphtheria toxin, cholera toxin, ricin or pseudomonas exotoxin, to a PSCA expressing cell; modulate PSCA activity;

30

to directly kill PSCA expressing cells; or in screens to identify competitive binding agents. For example, a PSCA inhibitory agent can be used to directly inhibit the growth of PSCA expressing cells whereas a PSCA binding agent can be used as a diagnostic agent.

5 There are multiple diagnostic uses of the invention. For example, the invention provides methods for diagnosing in a subject, e.g., an animal or human subject, a cancer associated with the presence of the PSCA protein. In one embodiment, the method comprises quantitatively determining the
10 number of PSCA protein in the sample (e.g., cell or biological fluid sample) using any one or combination of the antibodies of the invention. Then the number so determined can be compared with the amount in a sample from a normal subject. The presence of a measurably different amount (i.e., the number of PSCA in the test sample exceeds the number from a normal sample) in the samples indicating the presence of the cancer. PSCA is overexpressed on a cell when the number of PSCA in the test sample exceeds the number from a normal sample.

15 In another embodiment, diagnosis involves quantitatively determining in a sample from the subject the amount of RNA encoding the PSCA protein using the nucleic acid of the invention. The amount so determined can be compared with the amount of RNA in a sample from a normal subject. Once again, the presence of a measurable different amount indicating the presence of the cancer.

20 Additionally, the invention provides methods for monitoring the course of cancer (e.g., prostate cancer) or disorders associated with PSCA in a subject by measuring the amount of PSCA in a sample from the subject at various points in time. This is done for purposes of determining a change in the amount of PSCA in the sample e.g., to determine whether the change is a small
25 change in the amount or a large change, i.e., overexpression of PSCA. In one embodiment, the method comprises quantitatively determining in a first sample from the subject the presence of a PSCA protein and comparing the amount so determined with the amount present in a second sample from the subject, such samples being taken at different points in time, a difference in the amounts determined being indicative of the course of the cancer.

30

In another embodiment, monitoring is effected by quantitatively determining in a first sample from the subject the presence of a PSCA RNA and comparing the amount so determined with the amount present in a second sample from the subject, such samples being taken at different points in time, a difference in the amounts determined being indicative of the course of the cancer (e.g., prostate cancer).

As a further embodiment, the diseases or disorders associated with PSCA can be monitored in a sample by detecting an increase in or increased PSCA gene copy number. An increase in or increased PSCA gene copy number is important because it may correlate with poor outcome.

10

The sample can be from an animal or a human. Further, the sample can be a cell sample. For example, using the methods of the invention, prostate tissue, bladder tissue, neuroendocrine tissue, and bone (any tissue where prostate and bladder carcinomas can metastasize, e.g., node, lung, liver, pancreas) can be evaluated for the presence of cancer or metastatic lesion. Alternatively, the sample can be a biological fluid, e.g., urine, blood sera or plasma.

15

In accordance with the practice of the invention, detection can be effected by immunologic detection means involving histology, blotting, ELISA, and ELIFA. When the sample is a tissue or cell sample it can be formalin-fixed, paraffin-embedded or frozen.

20

The invention additionally provides methods of determining a difference in the amount and distribution of PSCA in tissue sections from a neoplastic tissue to be tested relative to the amount and distribution of PSCA in tissue sections from a normal tissue. In one embodiment, the method comprises contacting both the tissue to be tested and the normal tissue with a monoclonal antibody that specifically forms a complex with PSCA and thereby detecting the difference in the amount and distribution of PSCA.

25

Further, the invention provides a method for diagnosing a neoplastic or preneoplastic condition in a subject. This method comprises obtaining from the subject a sample of a tissue, detecting a

difference in the amount and distribution of PSCA in the using the method above, a distinct measurable difference being indicative of such neoplastic or preneoplastic condition.

5 In accordance with the practice of the invention, the antibody can be directed to the epitope to which any of the monoclonal antibodies of the invention is directed. Further, the tissue section can be from the bladder, prostate, bone, or muscle.

10 The invention also provides methods of detecting and quantitatively determining the concentration of PSCA in a biological fluid sample. In one embodiment the method comprises contacting a solid support with an excess of one or more monoclonal antibodies which forms (preferably specifically forms) a complex with PSCA under conditions permitting the monoclonal antibody to attach to the surface of the solid support. The resulting solid support to which the monoclonal antibody is attached is then contacted with a biological fluid sample so that the PSCA in the biological fluid binds to the antibody and forms a PSCA-antibody complex. The complexed
15 can be labeled directly or indirectly with a detectable marker. Alternatively, either the PSCA or the antibody can be labeled before the formation the complex. The complex can then be detected and quantitatively determined thereby detecting and quantitatively determining the concentration of PSCA in the biological fluid sample. A high concentration of PSCA in the sample relative to normal cells being indicative of a neoplastic or preneoplastic condition.

20

In accordance with the practice of the invention, the biological fluid includes but is not limited to tissue extract, urine, blood, serum, and phlegm. Further, the detectable marker includes but is not limited to an enzyme, biotin, a fluorophore, a chromophore, a heavy metal, a paramagnetic isotope, or a radioisotope.

25

Further, the invention provides a diagnostic kit comprising an antibody that recognizes and binds PSCA (an anti-PSCA antibody); and a conjugate of a detectable label and a specific binding partner of the anti-PSCA antibody. In accordance with the practice of the invention the label includes, but is not limited to, enzymes, radiolabels, chromophores and fluorescers.

PROSTATE CANCER IMMUNOTHERAPY

Since PSCA protein is overexpressed in many prostate tumors when compared to adjacent
5 normal glands, it is a target for prostate cancer immunotherapy. These immunotherapeutic
methods include the use of antibody therapy, in vivo vaccines, and ex vivo immunotherapy
approaches.

In one approach, the invention provides PSCA antibodies that may be used systemically to treat
10 prostate cancer. For example, unconjugated PSCA antibody (including monoclonal, polyclonal,
chimeric, humanized and fragments thereof (e.g., recombinant proteins)) may be introduced into a
patient such that the antibody binds to PSCA on prostate cancer cells and mediates growth inhibition
of such cells (including the destruction thereof), and the tumor, by mechanisms which may include
15 complement-mediated cytotoxicity, antibody-dependent cellular cytotoxicity, altering the physiologic
function of PSCA, and/or the inhibition of ligand binding or signal transduction pathways. In
addition to unconjugated PSCA antibodies, fragments thereof, and recombinant proteins of the
invention, PSCA antibodies conjugated to toxic agents such as ricin may also be used therapeutically
to deliver the toxic agent directly to PSCA-bearing prostate tumor cells and thereby destroy the
tumor.

20 Prostate cancer immunotherapy using PSCA antibodies may follow the teachings generated from
various approaches which have been successfully employed with respect to other types of cancer,
including but not limited to colon cancer (Arlen et al., 1998, Crit Rev Immunol 18: 133-138),
multiple myeloma (Ozaki et al., 1997, Blood 90: 3179-3186; Tsunenari et al., 1997, Blood 90: 2437-
25 2444), gastric cancer (Kasprzyk et al., 1992, Cancer Res 52: 2771-2776), B-cell lymphoma
(Funakoshi et al., 1996, J Immunother Emphasis Tumor Immunol 19: 93-101), leukemia (Zhong et al.,
1996, Leuk Res 20: 581-589), colorectal cancer (Moun et al., 1994, Cancer Res 54: 6160-6166);
Velders et al., 1995, Cancer Res 55: 4398-4403), and breast cancer (Shepard et al., 1991, J Clin
Immunol 11: 117-127).

For example, one way to apply antitumor monoclonal antibodies clinically is to administer them in unmodified form, using monoclonal antibodies of the invention which display antitumor activity (e.g., ADCC and CDC activity) and/or internalizing ability in vitro and/or in animal models (see, e.g. Hellstrom et al., Proc. Natl. Acad. Sci. USA 82:1499-1502 (1985). To detect ADCC and CDC activity monoclonal antibodies can be tested for lysing cultured ^{51}Cr -labeled tumor target cells over a 4-hour incubation period. Target cells are labeled with ^{51}Cr and then can be exposed for 4 hours to a combination of effector cells (in the form of human lymphocytes purified by the use of a lymphocyte-separation medium) and antibody, which is added in concentrations, e.g., varying between 0.1 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$. The release of ^{51}Cr from the target cells is measured as evidence of tumor-cell lysis (cytotoxicity). Controls include the incubation of target cells alone or with either lymphocytes or monoclonal antibody separately. The total amount of ^{51}Cr that can be released is measured and ADCC is calculated as the percent killing of target cells observed with monoclonal antibody plus effector cells as compared to target cells being incubated alone. The procedure for CDC is identical to the one used to detect ADCC except that human serum, as a source of complement, (diluted 1:3 to 1:6) is added in place of the effector cells.

The invention further provides vaccines formulated to contain a PSCA protein or fragment thereof. The use of a tumor antigen in a vaccine for generating humoral and cell-mediated immunity for use in anti-cancer therapy is well known in the art and has been employed in prostate cancer using human PSMA and rodent PAP immunogens (Hodge et al., 1995, *Int. J. Cancer* 63: 231-237; Fong et al., 1997, *J. Immunol.* 159: 3113-3117). Such methods can be readily practiced by employing a PSCA protein, or fragment thereof, or a PSCA-encoding nucleic acid molecule and recombinant vectors capable of expressing and appropriately presenting the PSCA immunogen.

For example, viral gene delivery systems may be used to deliver a PSCA-encoding nucleic acid molecule. Various viral gene delivery systems which can be used in the practice of this aspect of the invention include, but are not limited to, vaccinia, fowlpox, canarypox, adenovirus, influenza, poliovirus, adeno-associated virus, lentivirus, and sindbus virus (Restifo, 1996, *Curr. Opin. Immunol.* 8: 658-663). Non-viral delivery systems may also be employed by using naked DNA encoding a PSCA protein or fragment thereof introduced into the patient (e.g., intramuscularly) to induce an

anti-tumor response. In one embodiment, the full-length human PSCA cDNA may be employed. In another embodiment, PSCA nucleic acid molecules encoding specific cytotoxic T lymphocyte (CTL) epitopes may be employed. CTL epitopes can be determined using specific algorithms (e.g., Epimer, Brown University) to identify peptides within a PSCA protein which are capable of optimally binding to specified HLA alleles.

Various ex vivo strategies may also be employed. One approach involves the use of dendritic cells to present PSCA antigen to a patient's immune system. Dendritic cells express MHC class I and II, B7 costimulator, and IL-12, and are thus highly specialized antigen presenting cells. In prostate cancer, autologous dendritic cells pulsed with peptides of the prostate-specific membrane antigen (PSMA) are being used in a Phase I clinical trial to stimulate prostate cancer patients' immune systems (Tjoa et al., 1996, Prostate 28: 65-69; Murphy et al., 1996, Prostate 29: 371-380). Dendritic cells can be used to present PSCA peptides to T cells in the context of MHC class I and II molecules. In one embodiment, autologous dendritic cells are pulsed with PSCA peptides capable of binding to MHC molecules. In another embodiment, dendritic cells are pulsed with the complete PSCA protein. Yet another embodiment involves engineering the overexpression of the PSCA gene in dendritic cells using various implementing vectors known in the art, such as adenovirus (Arthur et al., 1997, Cancer Gene Ther. 4: 17-25), retrovirus (Henderson et al., 1996, Cancer Res. 56: 3763-3770), lentivirus, adeno-associated virus, DNA transfection (Ribas et al., 1997, Cancer Res. 57: 2865-2869), and tumor-derived RNA transfection (Ashley et al., 1997, J. Exp. Med. 186: 1177-1182).

Anti-idiotypic anti-PSCA antibodies can also be used in anti-cancer therapy as a vaccine for inducing an immune response to cells expressing a PSCA protein. Specifically, the generation of anti-idiotypic antibodies is well known in the art and can readily be adapted to generate anti-idiotypic anti-PSCA antibodies that mimic an epitope on a PSCA protein (see, for example, Wagner et al., 1997, Hybridoma 16: 33-40; Foon et al., 1995, J Clin Invest 96: 334-342; Herlyn et al., 1996, Cancer Immunol Immunother 43: 65-76). Such an anti-idiotypic antibody can be used in anti-idiotypic therapy as presently practiced with other anti-idiotypic antibodies directed against tumor antigens.

Genetic immunization methods may be employed to generate prophylactic or therapeutic humoral and cellular immune responses directed against cancer cells expressing PSCA. Using the PSCA-encoding DNA molecules described herein, constructs comprising DNA encoding a PSCA protein/immunogen and appropriate regulatory sequences may be injected directly into muscle or skin of an individual, such that the cells of the muscle or skin take-up the construct and express the encoded PSCA protein/immunogen. The PSCA protein/immunogen may be expressed as a cell surface protein or be secreted. Expression of the PSCA protein/immunogen results in the generation of prophylactic or therapeutic humoral and cellular immunity against prostate cancer. Various prophylactic and therapeutic genetic immunization techniques known in the art may be used (for review, see information and references published at internet address www.genweb.com).

The invention further provides methods for inhibiting cellular activity (e.g., cell proliferation, activation, or propagation) of a cell expressing multiple PSCA antigens on its cell surface. This method comprises reacting the immunoconjugates of the invention (e.g., a heterogenous or homogenous mixture) with the cell so that the PSCA antigens on the cell surface forms a complex with the immunoconjugates. The greater the number of PSCA antigens on the cell surface, the greater the number of PSCA-antibody complexes can be used. The greater the number of PSCA-antibody complexes the greater the cellular activity that is inhibited. A subject with a neoplastic or preneoplastic condition can be treated in accordance with this method when the inhibition of cellular activity results in cell death.

A heterogenous mixture includes PSCA antibodies that recognize different or the same epitope, each antibody being conjugated to the same or different therapeutic agent. A homogenous mixture includes antibodies that recognize the same epitope, each antibody being conjugated to the same therapeutic agent.

The invention further provides methods for inhibiting the biological activity of PSCA by blocking PSCA from binding its ligand. The methods comprises contacting an amount of PSCA with an antibody or immunoconjugate of the invention under conditions that permit a PSCA-

immunoconjugate or PSCA-antibody complex thereby blocking PSCA from binding its ligand and inhibiting the activity of PSCA.

5 In another embodiment, the invention provides methods for selectively inhibiting a cell expressing PSCA antigen by reacting any one or a combination of the immunoconjugates of the invention with the cell in an amount sufficient to inhibit the cell. Such amounts include an amount to kill the cell or an amount sufficient to inhibit cell growth or proliferation. As discussed supra the dose and dosage regimen will depend on the nature of the disease or disorder to be treated associated with PSCA, its population, the site to which the antibodies are to be directed, the characteristics of the
10 particular immunotoxin, and the patient. For example, the amount of immunoconjugate can be in the range of 0.1 to 10 mg/kg of patient weight.

METHODS FOR IDENTIFYING PSCA PROTEINS AND PSCA GENES AND RNA

15 The invention provides methods for identifying cells, tissues or organisms expressing a PSCA protein or a PSCA gene. Such methods can be used to diagnose the presence of cells or an organism that expresses a PSCA protein *in vivo* or *in vitro*. The methods of the present invention are particularly useful in the determining the presence of cells that mediate pathological conditions of the prostate. Specifically, the presence of a PSCA protein can be identified by determining whether a
20 PSCA protein, or nucleic acid encoding a PSCA protein, is expressed. The expression of a PSCA protein can be used as a means for diagnosing the presence of cells, tissues or an organism that expresses a PSCA protein or gene.

A variety of immunological and molecular genetic techniques can be used to determine if a PSCA
25 protein is expressed/produced in a particular cell or sample. In general, an extract containing nucleic acid molecules or an extract containing proteins is prepared. The extract is then assayed to determine whether a PSCA protein, or a PSCA-encoding nucleic acid molecule, is produced in the cell.

Various polynucleotide-based detection methods well known in the art may be employed for the
30 detection of PSCA-encoding nucleic acid molecules and for the detection of PSCA expressing cells

in a biological specimen. For example, RT-PCR methods may be used to selectively amplify a PSCA mRNA or fragment thereof, and such methods may be employed to identify cells expressing PSCA, as described in Example 1 below. In a particular embodiment, RT-PCR is used to detect micrometastatic prostate cancer cells or circulating prostate cancer cells. Various other amplification type detection methods, such as, for example, branched DNA methods, and various well known hybridization assays using DNA or RNA probes may also be used for the detection of PSCA-encoding polynucleotides or PSCA expressing cells.

Various methods for the detection of proteins are well known in the art and may be employed for the detection of PSCA proteins and PSCA expressing cells. To perform a diagnostic test based on proteins, a suitable protein sample is obtained and prepared using conventional techniques. Protein samples can be prepared, for example, simply by boiling a sample with SDS. The extracted protein can then be analyzed to determine the presence of a PSCA protein using known methods. For example, the presence of specific sized or charged variants of a protein can be identified using mobility in an electric field. Alternatively, antibodies can be used for detection purposes. A skilled artisan can readily adapt known protein analytical methods to determine if a sample contains a PSCA protein.

Alternatively, PSCA expression can also be used in methods to identify agents that decrease the level of expression of the PSCA gene. For example, cells or tissues expressing a PSCA protein can be contacted with a test agent to determine the effects of the agent on PSCA expression. Agents that activate PSCA expression can be used as an agonist of PSCA activity whereas agents that decrease PSCA expression can be used as an antagonist of PSCA activity.

PSCA PROMOTER AND OTHER EXPRESSION REGULATORY ELEMENTS

The invention further provides expression control sequences found 5' of the of the newly identified *PSCA* gene in a form that can be used to generate expression vectors and transgenic animals. Specifically, the *PSCA* expression control elements, such as the PSCA promoter that can readily be identified as being 5' from the ATG start codon in the *PSCA* gene, and can be used to direct the

expression of an operably linked protein encoding DNA sequence. Since PSCA expression is predominantly expressed in prostate cells, the expression control elements are particularly useful in directing the expression of an introduced transgene in a tissue specific fashion. A skilled artisan can readily use the PSCA gene promoter and other regulatory elements in expression vectors using methods known in the art.

In eukaryotic cells, the regulatory sequences can be found upstream, downstream and within the coding region of the gene. The eukaryotic regulatory sequences comprise a promoter sequence and sometimes at least one enhancer sequence. In a typical eukaryotic gene, the promoter sequence resides upstream and proximal to the coding region of the gene, and must be oriented in one direction to control expression of the gene. In a typical eukaryotic gene, the enhancer sequences can reside in the upstream, downstream and even within the coding region of the gene, and can be oriented in either direction to enhance or suppress expression of the gene.

The present invention provides a DNA fragment containing 9 kb of sequences upstream of the PSCA coding region. The ability of this PSCA fragment to drive expression of an operatively linked transgene has been tested using a series of chimeric reporter constructs transfected into cells. The chimeric reporter constructs demonstrate an expression pattern similar to that of native endogenous PSCA, and the PSCA fragment drives expression of the transgene when linked in the forward orientation. Thus, this PSCA fragment comprises a PSCA upstream regulatory region that exhibits promoter-like activity.

PSCA transcripts are also present at a significantly higher level in prostate tumor cells but not in benign prostatic hyperplasia. Thus PSCA transcripts are detectable in a prostate-predominant manner, and are detectable at a higher level in prostate tumor samples. The significantly higher level of PSCA transcripts, or over-expression as is known in the art, can be determined by measuring and comparing the amount of detectable PSCA transcripts in a normal prostate with a prostate tumor sample. This comparison can be performed by methods well known in the art, including Northern analysis and RT-PCR assays, and the differences in transcript levels can be quantitated. Thus, the presence of a measurably different amount of PSCA transcripts (i.e., the number of PSCA

transcripts in the test sample exceeds the number from a normal sample) in the samples can be used to indicate the presence of prostate cancer.

5 The pattern of PSCA transcript and protein accumulation is known, and the PSCA upstream regulatory region has been isolated and characterized. A series of chimeric constructs comprising the PSCA upstream regulatory region operatively linked to a transgene has been tested. The PSCA upstream regulatory region drives expression of the transgene in various prostate cells and cell lines, and in bladder, and to a lesser extent in kidney. Thus, the PSCA upstream region drives expression of a transgene in a prostate-predominant manner.

10 In preferred embodiments, DNA fragments of 9kb, 6kb, 3kb, and 1kb derived from the 5' upstream region of the PSCA gene, as shown in Figure 42, were produced by techniques described herein. The 9kb PSCA upstream region (pEGFP—PSCA) is involved with gene regulatory activity and was deposited on May 17, 1999 with the American Type Culture Collection (ATCC),
15 12301 Parklawn Drive, Rockville, MD 20852 and has there been identified as follows ATCC No. PTA-80. The 9 kb fragment was obtained by amplification using a T7 primer and RlhPSCA3'-5' (5'-gggaattcgcacagccttcagggtc-3').

20 USES OF THE FRAGMENT HAVING GENE REGULATORY ACTIVITY

This invention provides methods (e.g., gene therapy methods) for targeting a gene-of-interest to a diseased prostate cancer cells/site so that the protein encoded by the gene can be expressed thereby directly or indirectly ameliorating the diseased state.

25 A susceptible cell is introduced with an expression vector that expresses a transgene (e.g., a therapeutic gene) under the control of a PSCA upstream region having significantly increased gene expression activity in prostate tumor cells. The use of an expression vector that expresses a therapeutic gene predominantly in prostate tumor cells will allow expression of the therapeutic genes in target cell, such as prostate tumor cells.

After infecting a susceptible cell, a transgene (e.g., a therapeutic gene) is driven by a PSCA upstream region having increased gene expression activity in a vector, that expresses the protein encoded by the transgene. The use of a fairly specific prostate specific gene vector will allow selective
5 expression of the specific genes in target cells, e.g., prostate cancer cells.

PSCA regions having increased gene expression activity may be modified, e.g., by sequence mutations, deletions, and insertions, so as to produce derivative molecules. Modifications include multiplying the number of sequences that can bind prostate cell specific regulatory proteins and
10 deleting sequences that are nonfunctional in the PSCA region having gene expression activity. Other modifications include adding enhancers thereby improving the efficiency of the PSCA region having promoter activity. Enhancers may function in a position-independent manner and can be located upstream, within or downstream of the transcribed region.

15 Derivative molecules would retain the functional property of the PSCA upstream region having increased gene expression activity, namely, the molecule having such substitutions will still permit substantially prostate tissue specific expression of a gene of interest located 3' to the fragment. Modification is permitted so long as the derivative molecules retain its ability to drive gene expression in a substantially prostate specific manner compared to a PSCA fragment having
20 promoter activity alone.

In a preferred embodiment, a vector was constructed by inserting a heterologous sequence (therapeutic gene) downstream of the PSCA upstream region having promoter activity.

25 Examples of therapeutic genes include suicide genes. These are genes sequences the expression of which produces a protein or agent that inhibits prostate tumor cell growth or prostate tumor cell death. Suicide genes include genes encoding enzymes (e.g., prodrug enzymes), oncogenes, tumor suppressor genes, genes encoding toxins, genes encoding cytokines, or a gene encoding oncostatin. The purpose of the therapeutic gene is to inhibit the growth of or kill prostate cancer cell or produce

cytokines or other cytotoxic agents which directly or indirectly inhibit the growth of or kill prostate cancer cells.

Suitable prodrug enzymes include thymidine kinase (TK), xanthine-guanine
5 phosphoribosyltransferase (GPT) gene from E. Coli or E. Coli cytosine deaminase (CD), or hypoxanthine phosphoribosyl transferase (HPRT).

Suitable oncogenes and tumor suppressor genes include neu, EGF, ras (including H, K, and N ras), p53, Retinoblastoma tumor suppressor gene (Rb), Wilm's Tumor Gene Product, Phosphotyrosine
10 Phosphatase (PTPase), and nm23. Suitable toxins include Pseudomonas exotoxin A and S; diphtheria toxin (DT); E. coli LT toxins, Shiga toxin, Shiga-like toxins (SLT-1, -2), ricin, abrin, supporin, and gelonin.

Suitable cytokines include interferons, GM-CSF interleukins, tumor necrosis factor (TNF) (Wong G,
15 et al., Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science 1985; 228:810); WO9323034 (1993); Horisberger MA, et al., Cloning and sequence analyses of cDNAs for interferon- and virus-induced human Mx proteins reveal that they contain putative guanine nucleotide-binding sites: functional study of the corresponding gene promoter. Journal of Virology, 1990 Mar, 64(3):1171-81; Li YP et al.,
20 Proinflammatory cytokines tumor necrosis factor-alpha and IL-6, but not IL-1, down-regulate the osteocalcin gene promoter. Journal of Immunology, 1992 Feb 1, 148(3):788-94; Pizarro TT, et al. Induction of TNF alpha and TNF beta gene expression in rat cardiac transplants during allograft rejection. Transplantation, 1993 Aug, 56(2):399-404). (Breviario F, et al., Interleukin-1-inducible genes in endothelial cells. Cloning of a new gene related to C-reactive protein and serum amyloid P
25 component. Journal of Biological Chemistry, 1992 Nov 5, 267(31):22190-7; Espinoza-Delgado I, et al., Regulation of IL-2 receptor subunit genes in human monocytes. Differential effects of IL-2 and IFN-gamma. Journal of Immunology, 1992 Nov 1, 149(9):2961-8; Algate PA, et al., Regulation of the interleukin-3 (IL-3) receptor by IL-3 in the fetal liver-derived FL5.12 cell line. Blood, 1994 May 1, 83(9):2459-68; Cluitmans FH, et al., IL-4 down-regulates IL-2-, IL-3-, and GM-CSF-induced
30 cytokine gene expression in peripheral blood monocytes. Annals of Hematology, 1994 Jun,

- 68(6):293-8; Lagoo, AS, et al., IL-2, IL-4, and IFN-gamma gene expression versus secretion in superantigen-activated T cells. Distinct requirement for costimulatory signals through adhesion molecules. Journal of Immunology, 1994 Feb 15, 152(4):1641-52; Martinez OM, et al., IL-2 and IL-5 gene expression in response to alloantigen in liver allograft recipients and in vitro. Transplantation, 1993 May, 55(5):1159-66; Pang G, et al., GM-CSF, IL-1 alpha, IL-1 beta, IL-6, IL-8, IL-10, ICAM-1 and VCAM-1 gene expression and cytokine production in human duodenal fibroblasts stimulated with lipopolysaccharide, IL-1 alpha and TNF-alpha. Clinical and Experimental Immunology, 1994 Jun, 96(3):437-43; Ulich TR, et al., Endotoxin-induced cytokine gene expression in vivo. III. IL-6 mRNA and serum protein expression and the in vivo hematologic effects of IL-6. Journal of Immunology, 1991 Apr 1, 146(7):2316-23; Mauviel A, et al., Leukoregulin, a T cell-derived cytokine, induces IL-8 gene expression and secretion in human skin fibroblasts. Demonstration and secretion in human skin fibroblasts. Demonstration of enhanced NF-kappa B binding and NF-kappa B-driven promoter activity. Journal of Immunology, 1992 Nov 1, 149(9):2969-76).
- 15 Growth factors include Transforming Growth Factor- α (TGF α) and β (TGF β), cytokine colony stimulating factors (Shimane M, et al., Molecular cloning and characterization of G-CSF induced gene cDNA. Biochemical and Biophysical Research Communications, 1994 Feb 28, 199(1):26-32; Kay AB, et al., Messenger RNA expression of the cytokine gene cluster, interleukin 3 (IL-3), IL-4, IL-5, and granulocyte/macrophage colony-stimulating factor, in allergen-induced late-phase
- 20 cutaneous reactions in atopic subjects. Journal of Experimental Medicine, 1991 Mar 1, 173(3):775-8; de Wit H, et al., Differential regulation of M-CSF and IL-6 gene expression in monocytic cells. British Journal of Haematology, 1994 Feb, 86(2):259-64; Sprecher E, et al., Detection of IL-1 beta, TNF-alpha, and IL-6 gene transcription by the polymerase chain reaction in keratinocytes, Langerhans cells and peritoneal exudate cells during infection with herpes simplex virus-1. Archives
- 25 of Virology, 1992, 126(1-4):253-69).

Vectors suitable for use in the methods of the present invention are viral vectors including adenoviruses, lentivirus, retroviral vectors, adeno-associated viral (AAV) vectors.

- Preferably, the viral vector selected should meet the following criteria: 1) the vector must be able to infect the tumor cells and thus viral vectors having an appropriate host range must be selected; 2) the transferred gene should be capable of persisting and being expressed in a cell for an extended period of time; and 3) the vector should be safe to the host and cause minimal cell transformation.
- 5 Retroviral vectors and adenoviruses offer an efficient, useful, and presently the best-characterized means of introducing and expressing foreign genes efficiently in mammalian cells. These vectors have very broad host and cell type ranges, express genes stably and efficiently. The safety of these vectors has been proved by many research groups. In fact many are in clinical trials.
- 10 Other virus vectors that may be used for gene transfer into cells for correction of disorders include retroviruses such as Moloney murine leukemia virus (MoMuLV); papovaviruses such as JC, SV40, polyoma; Epstein-Barr Virus (EBV); papilloma viruses, e.g. bovine papilloma virus type I (BPV); vaccinia and poliovirus and other human and animal viruses.
- 15 Adenoviruses have several properties that make them attractive as cloning vehicles (Bachettis et al.: Transfer of gene for thymidine kinase-deficient human cells by purified herpes simplex viral DNA. PNAS USA, 1977 74:1590; Berkner, K.L.: Development of adenovirus vectors for expression of heterologous genes. Biotechniques, 1988 6:616; Ghosh-Choudhury G, et al., Human adenovirus cloning vectors based on infectious bacterial plasmids. Gene 1986; 50:161; Hag-Ahmand Y, et al.,
- 20 Development of a helper-independent human adenovirus vector and its use in the transfer of the herpes simplex virus thymidine kinase gene. J Virol 1986; 57:257; Rosenfeld M, et al., Adenovirus-mediated transfer of a recombinant α_1 -antitrypsin gene to the lung epithelium in vivo. Science 1991; 252:431).
- 25 For example, adenoviruses possess an intermediate sized genome that replicates in cellular nuclei; many serotypes are clinically innocuous; adenovirus genomes appear to be stable despite insertion of foreign genes; foreign genes appear to be maintained without loss or rearrangement; and adenoviruses can be used as high level transient expression vectors with an expression period up to 4 weeks to several months. Extensive biochemical and genetic studies suggest that it is possible to
- 30 substitute up to 7-7.5 kb of heterologous sequences for native adenovirus sequences generating

viable, conditional, helper-independent vectors (Kaufman R.J.; identification of the component necessary for adenovirus translational control and their utilization in cDNA expression vectors. PNAS USA, 1985 82:689).

- 5 AAV is a small human parvovirus with a single stranded DNA genome of approximately 5 kb. This virus can be propagated as an integrated provirus in several human cell types. AAV vectors have several advantage for human gene therapy. For example, they are trophic for human cells but can also infect other mammalian cells; (2) no disease has been associated with AAV in humans or other animals; (3) integrated AAV genomes appear stable in their host cells; (4) there is no evidence that
10 integration of AAV alters expression of host genes or promoters or promotes their rearrangement; (5) introduce genes can be rescued from the host cell by infection with a helper virus such as adenovirus.

- HSV-1 vector system facilitates introduction of virtually any gene into non-mitotic cells (Geller et al.
15 an efficient deletion mutant packaging system for a defective herpes simplex virus vectors: Potential applications to human gene therapy and neuronal physiology. PNAS USA, 1990 87:8950).

Another vector for mammalian gene transfer is the bovine papilloma virus-based vector (Sarver N, et al., Bovine papilloma virus DNA: A novel eukaryotic cloning vector. Mol Cell Biol 1981; 1:486).

- 20 Vaccinia and other poxvirus-based vectors provide a mammalian gene transfer system. Vaccinia virus is a large double-stranded DNA virus of 120 kilodaltons (kd) genomic size (Panicali D, et al., Construction of poxvirus as cloning vectors: Insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccine virus. Proc Natl Acad Sci USA 1982; 79:4927;
25 Smith et al. infectious vaccinia virus recombinants that express hepatitis B virus surface antigens. Nature, 1983 302:490.)

Retroviruses are packages designed to insert viral genes into host cells (Guild B, et al., Development of retrovirus vectors useful for expressing genes in cultured murine embryonic cells and

hematopoietic cells in vivo. J Virol 1988; 62:795; Hock RA, et al., Retrovirus mediated transfer and expression of drug resistance genes in human hemopoietic progenitor cells. Nature 1986; 320:275).

5 The basic retrovirus consists of two identical strands of RNA packaged in a proviral protein. The core surrounded by a protective coat called the envelope, which is derived from the membrane of the previous host but modified with glycoproteins contributed by the virus.

10 Preferably, for treating defects, disease or damage of cells in the prostate, vectors of the invention include a therapeutic gene or transgenes, for example a gene encoding TK. The genetically modified vectors are administered into the prostate to treat defects, disease such as prostate cancer by introducing a therapeutic gene product or products into the prostate that enhance the production of endogenous molecules that have ameliorative effects in vivo.

15 The basic tasks in this embodiment of the present method of the invention are isolating the gene of interest, attaching it to a fragment having gene regulatory activity, selecting the proper vector vehicle to deliver the gene of interest to the body, administering the vector having the gene of interest into the body, and achieving appropriate expression of the gene of interest to a target cell. The present invention provides packaging the cloned genes, i.e. the genes of interest, in such a way that they can be injected directly into the bloodstream or relevant organs of patients who need them. The
20 packaging will protect the foreign DNA from elimination by the immune system and direct it to appropriate tissues or cells.

Along with the human or animal gene of interest another gene, e.g., a selectable marker, can be inserted that will allow easy identification of cells that have incorporated the modified retrovirus. The critical focus on the process of gene therapy is that the new gene must be expressed in target
25 cells at an appropriate level with a satisfactory duration of expression.

The methods described below to modify vectors and administering such modified vectors into the prostate are merely for purposes of illustration and are typical of those that might be used. However, other procedures may also be employed, as is understood in the art.

Most of the techniques used to construct vectors and the like are widely practiced in the art, and most practitioners are familiar with the standard resource materials which describe specific conditions and procedures. However, for convenience, the following paragraphs may serve as a guideline.

5 GENERAL METHODS FOR VECTOR CONSTRUCTION

Construction of suitable vectors containing the desired therapeutic gene coding and control sequences employs standard ligation and restriction techniques, which are well understood in the art (see Maniatis et al., in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1982)). Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

Site-specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes (See, e.g. New England Biolabs Product Catalog). In general, about 1 µg of plasmid or DNA sequences is cleaved by one unit of enzyme in about 20 µl of buffer solution. Typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate.

Incubation times of about one hour to two hours at about 37°C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in Methods in Enzymology 65:499-560 (1980).

Restriction cleaved fragments may be blunt ended by treating with the large fragment of E. coli DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 min at 20°C to 25°C in 50 mM Tris (pH 7.6) 50 mM NaCl, 6 mM

MgCl₂, 6 mM DTT and 5-10 μM dNTPs. The Klenow fragment fills in at 5' sticky ends but chews back protruding 3' single strands, even though the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the dNTPs, or with selected dNTPs, within the limitations dictated by the nature of the sticky ends. After treatment with Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease or Bal-31 results in hydrolysis of any single-stranded portion.

Ligations are performed in 10-50 μl volumes under the following standard conditions and temperatures using T4 DNA ligase. Ligation protocols are standard (D. Goeddel (ed.) *Gene Expression Technology: Methods in Enzymology* (1991)).

In vector construction employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIP) in order to remove the 5' phosphate and prevent religation of the vector. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments.

Suitable vectors include viral vector systems e.g. ADV, RV, and AAV (R.J. Kaufman "Vectors used for expression in mammalian cells" in *Gene Expression Technology*, edited by D.V. Goeddel (1991).

Many methods for inserting functional DNA transgenes into cells are known in the art. For example, non-vector methods include nonviral physical transfection of DNA into cells; for example, microinjection (DePamphilis et al., *BioTechnique* 6:662-680 (1988)); liposomal mediated transfection (Felgner et al., *Proc. Natl. Acad. Sci. USA*, 84:7413-7417 (1987), Felgner and Holm, *Focus* 11:21-25 (1989) and Felgner et al., *Proc. West. Pharmacol. Soc.* 32: 115-121 (1989)) and other methods known in the art.

ADMINISTRATION OF MODIFIED VECTORS INTO SUBJECT

One way to get DNA into a target cell is to put it inside a membrane bound sac or vesicle such as a spheroplast or liposome, or by calcium phosphate precipitation (CaPO₄) (Graham F. and Van der Eb,

A., Virology 52:456 1973; Schaefer-Ridder M., et al., Liposomes as gene carriers: Efficient transduction of mouse L cells by thymidine kinase gene. Science 1982; 215:166; Stavridis JC, et al., Construction of transferrin-coated liposomes for in vivo transport of exogenous DNA to bone marrow erythroblasts in rabbits. Exp Cell Res 1986; 164:568-572).

5

A vesicle can be constructed in such a way that its membrane will fuse with the outer membrane of a target cell. The vector of the invention in vesicles can home into the prostate cells.

10

The spheroplasts are maintained in high ionic strength buffer until they can be fused through the mammalian target cell using fusogens such as polyethylene glycol.

15

Liposomes are artificial phospholipid vesicles. Vesicles range in size from 0.2 to 4.0 micrometers and can entrap 10% to 40% of an aqueous buffer containing macromolecules. The liposomes protect the DNA from nucleases and facilitate its introduction into target cells. Transfection can also occur through electroporation.

20

Before administration, the modified vectors are suspended in complete PBS at a selected density for injection. In addition to PBS, any osmotically balanced solution which is physiologically compatible with the subject may be used to suspend and inject the modified vectors into the host.

25

For injection, the cell suspension is drawn up into the syringe and administered to anesthetized recipients. Multiple injections may be made using this procedure. The viral suspension procedure thus permits administration of genetically modified vectors to any predetermined site in the prostate, is relatively non-traumatic, allows multiple administrations simultaneously in several different sites or the same site using the same viral suspension. Multiple injections may consist of a mixture of therapeutic genes.

USES OF THE MODIFIED VECTORS

The present invention provides methods for maintaining and increasing expression of therapeutic genes using a fragment having expression activity.

5

The methods of the invention are exemplified by embodiments in which modified vectors carrying a therapeutic gene are injected into a subject.

10 In a first embodiment a protein product is expressed comprising growing the host vector system of the invention so as to produce the protein in the host and recovering the protein so produced. This method permits the expression of genes of interest in both unicellular and multicellular organisms. For example, in an in vitro assay, prostate cells having the vector of the invention comprising a gene of interest (e.g., the ras gene) may be used in microtiter wells as an unlimited for the ras gene product. A sample from a subject would be added to the wells to detect the presence of antibodies
15 directed against the ras gene. This assay can aid in the quantitative and qualitative determination of the presence of ras antibodies in the sample for the clinical assessment of whether the subject's immune system is combatting the disease associated with elevated levels of ras.

20 In a second embodiment metastatic prostate cancer is treated via gene therapy, i.e., the correction of a disease phenotype in vivo through the use of the nucleic acid molecules of the invention.

In accordance with the practice of this invention, the subject of the gene therapy may be a human, equine, porcine, bovine, murine, canine, feline, or avian subject. Other mammals are also included in this invention.

25

The most effective mode of administration and dosage regimen for the molecules of the present invention depends upon the exact location of the prostate tumor being treated, the severity and course of the cancer, the subject's health and response to treatment and the judgment of the treating physician. Accordingly, the dosages of the molecules should be titrated to the individual subject.

The molecules may be delivered directly or indirectly via another cell, autologous cells are preferred, but heterologous cells are encompassed within the scope of the invention.

5 The interrelationship of dosages for animals of various sizes and species and humans based on mg/m^2 of surface area is described by Freireich, E.J., et al. Cancer Chemother., Rep. 50 (4): 219-244 (1966). Adjustments in the dosage regimen may be made to optimize the tumor cell growth inhibiting and killing response, e.g., doses may be divided and administered on a daily basis or the dose reduced proportionally depending upon the situation (e.g., several divided dose may be administered daily or proportionally reduced depending on the specific therapeutic situation).

10

It would be clear that the dose of the molecules of the invention required to achieve treatment may be further modified with schedule optimization.

GENERATION OF TRANSGENIC ANIMALS

15

Another aspect of the invention provides transgenic non-human mammals comprising *PSCA* nucleic acids. For example, in one application, *PSCA*-deficient non-human animals can be generated using standard knock-out procedures to inactivate a *PSCA* homologue or, if such animals are non-viable, inducible *PSCA* homologue antisense molecules can be used to regulate *PSCA* homologue activity/expression. Alternatively, an animal can be altered so as to contain a human *PSCA*-encoding nucleic acid molecule or an antisense-*PSCA* expression unit that directs the expression of *PSCA* protein or the antisense molecule in a tissue specific fashion. In such uses, a non-human mammal, for example a mouse or a rat, is generated in which the expression of the *PSCA* homologue gene is altered by inactivation or activation and/or replaced by a human *PSCA* gene. This can be accomplished using a variety of art-known procedures such as targeted recombination. Once generated, the *PSCA* homologue deficient animal, the animal that expresses *PSCA* (human or homologue) in a tissue specific manner, or an animal that expresses an antisense molecule can be used to (1) identify biological and pathological processes mediated by the *PSCA* protein, (2) identify proteins and other genes that interact with the *PSCA* proteins, (3) identify agents that can be

20

25

exogenously supplied to overcome a PSCA protein deficiency and (4) serve as an appropriate screen for identifying mutations within the *PSCA* gene that increase or decrease activity.

For example, it is possible to generate transgenic mice expressing the human minigene encoding PSCA in a tissue specific-fashion and test the effect of over-expression of the protein in tissues and cells that normally do not contain the PSCA protein. This strategy has been successfully used for other genes, namely *bcl-2* (Veis et al. Cell 1993 75:229). Such an approach can readily be applied to the PSCA protein/gene and can be used to address the issue of a potential beneficial or detrimental effect of the PSCA proteins in a specific tissue.

Further, in another embodiment, the invention provides a transgenic animal having germ and somatic cells comprising an oncogene which is linked to a PSCA upstream region effective for the expression of said gene in the tissues of said mouse for the promotion of a cancer associated with the oncogene, thereby producing a mouse model of that cancer

COMPOSITIONS

The invention provides a pharmaceutical composition comprising a PSCA nucleic acid molecule of the invention or an expression vector encoding a PSCA protein or encoding a fragment thereof and, optionally, a suitable carrier. The invention additionally provides a pharmaceutical composition comprising an antibody or fragment thereof which recognizes and binds a PSCA protein. In one embodiment, the antibody or fragment thereof is conjugated or linked to a therapeutic drug or a cytotoxic agent.

Suitable carriers for pharmaceutical compositions include any material which when combined with the nucleic acid or other molecule of the invention retains the molecule's activity and is non-reactive with the subject's immune systems. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Other carriers may also include

sterile solutions, tablets including coated tablets and capsules. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients.

5 Compositions comprising such carriers are formulated by well known conventional methods. Such compositions may also be formulated within various lipid compositions, such as, for example, liposomes as well as in various polymeric compositions, such as polymer microspheres.

The invention also provides a diagnostic composition comprising a PSCA nucleic acid molecule, a probe that specifically hybridizes to a nucleic acid molecule of the invention or to any part thereof, or a PSCA antibody or fragment thereof. The nucleic acid molecule, the probe or the antibody or fragment thereof can be labeled with a detectable marker. Examples of a detectable marker include, but are not limited to, a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator or an enzyme. Further, the invention

10 provides a diagnostic composition comprising a PSCA-specific primer pair capable of amplifying PSCA-encoding sequences using polymerase chain reaction methodologies, such as RT-PCR.

15

EXAMPLES

20 Example 1: Identification And Molecular Characterization Of A Novel Prostate Cell Surface Antigen (PSCA)

MATERIALS AND METHODS

25 LAPC-4 Xenografts: LAPC-4 xenografts were generated as described in Klein et al, 1997, Nature Med. 3: 402-408.

RDA, Northern Analysis and RT-PCR: Representational difference analysis of androgen dependent and independent LAPC-4 tumors was performed as previously described (Braun et al.,

30 1995, Mol. Cell. Biol. 15: 4623-4630). Total RNA was isolated using Ultraspec^R RNA isolation systems (Biotecx, Houston, TX) according to the manufacturer's instructions. Northern filters

were probed with a 660bp RDA fragment corresponding to the coding sequence and part of the 3' untranslated sequence of PSCA or a ~400bp fragment of PSA. The human multiple tissue blot was obtained from Clontech and probed as specified. For reverse transcriptase (RT)-PCR analysis, first strand cDNA was synthesized from total RNA using the GeneAmp RNA PCR core kit (Perkin Elmer-Roche, New Jersey). For RT-PCR of human PSCA transcripts, primers 5'-tgcttgccctgttgatggcag- and 3'-ccagagcagcaggccgagtga- were used to amplify a ~320 bp fragment. Thermal cycling was performed by 25-25 cycles of 95° for 30 sec, 60° for 30sec and 72° for 1 min, followed by extension at 72° for 10 min. Primers for GAPDH (Clontech) were used as controls. For mouse PSCA, the primers used were 5'-ttctctgctggccacctac- and 3'-gcagctcatcccttcacaat-.

In Situ Hybridization Assay for PSCA mRNA: For mRNA in situ hybridization, recombinant plasmid pCR II (1 ug, Invitrogen, San Diego, CA) containing the full-length PSCA gene was linearized to generate sense and antisense digoxigenin labeled riboprobes. In situ hybridization was performed on an automated instrument (Ventana Gen II, Ventana Medical Systems) as previously described (Magi-Galluzzi et al., 1997, Lab. Invest. 76: 37-43). Prostate specimens were obtained from a previously described database which has been expanded to ~130 specimens (Magi-Galluzzi et al., supra). Slides were read and scored by two pathologists in a blinded fashion. Scores of 0-3 were assigned according to the percentage of positive cells (0 = 0%; 1 = <25%; 2 = 25-50%; 3 = >50%) and the intensity of staining (0 = 0; 1 = 1+; 2 = 2+; 3 = 3+). The two scores were multiplied to give an overall score of 0-9.

RESULTS

Human PSCA cDNA: Representational Difference Analysis (RDA), a PCR-based subtractive hybridization technique, was used to compare gene expression between hormone dependent and hormone independent variants of a human prostate cancer xenograft (LAPC-4) and to isolate cDNAs upregulated in the androgen-independent LAPC-4 subline. Multiple genes were cloned, sequenced, and checked for differential expression. One 660bp fragment (clone #15) was identified which was found to be highly overexpressed in xenograft tumors when compared with normal prostate.

Comparison of the expression of this clone to that of PSA in normal prostate and xenograft tumors suggested that clone #15 was relatively cancer specific (FIG. 9).

Sequence analysis revealed that clone #15 had no exact match in the databases, but shared 30% nucleotide homology with stem cell antigen 2, a member of the Thy-1/Ly-6 superfamily of glycosylphosphatidylinositol (GPI)-anchored cell surface antigens. Clone #15 encodes a 123 amino acid protein which is 30% identical to SCA-2 (also called RIG-E) and contains a number of highly conserved cysteine residues characteristic of the Ly-6/Thy-1 gene family (FIG. 3). Consistent with its homology to a family of GPI-anchored proteins, clone #15 contains both an amino-terminal hydrophobic signal sequence and a carboxyl-terminal stretch of hydrophobic amino acids preceded by a group of small amino acids defining a cleavage/binding site for GPI linkage (Udenfriend and Kodukula, 1995, Ann. Rev. Biochem. 64: 563-591). It also contains four predicted N-glycosylation sites. Because of its strong homology to the stem cell antigen-2, clone #15 was renamed prostate stem cell antigen (PSCA). 5' and 3' PCR RACE analysis was then performed using cDNA obtained from the LAPC-4 androgen independent xenograft and the full length cDNA nucleotide sequence (including the coding and untranslated regions) was obtained. The nucleotide sequence of the full length cDNA encoding human PSCA is shown in FIG. 1A and the translated amino acid sequence is shown in FIG. 1B and in FIG. 3.

PSCA is expressed in prostate cells: The distribution of PSCA mRNA in normal human tissues was examined by Northern blot analysis. The results, shown in FIG. 9B, demonstrate that PSCA is expressed predominantly in prostate, with a lower level of expression present in placenta. Small amounts of mRNA can be detected in kidney and small intestine after prolonged exposure and at approximately 1/100th of the level seen in prostate tissue. RT-PCR analysis of PSCA expression in normal human tissues also demonstrates that PSCA expression is restricted. In a panel of normal tissues, high level PSCA mRNA expression was detected in prostate, with significant expression detected in placenta and tonsils (FIG. 7A). RT-PCR analysis of PSCA mRNA expression in a variety of prostate cancer xenografts prostate cancer cell lines and other cell lines, and normal prostate showed high level expression restricted to normal prostate, the LAPC-4 and LAPC-9 prostate cancer xenografts, and the ovarian cancer cell line A431 (FIG. 7B).

The major PSCA transcript in normal prostate is ~1kb (FIG. 9B). Mouse PSCA expression was analyzed by RT-PCR in mouse spleen, liver, lung, prostate, kidney and testis. Like human PSCA, murine PSCA is expressed predominantly in prostate. Expression can also be detected in kidney at a level similar to that seen for placenta in human tissues.

The expression of PSCA, PSMA and PSA in prostate cancer cell lines and xenografts was compared by Northern blot analysis. The results shown in FIG. 10 demonstrate high level prostate cancer specific expression of both PSCA and PSMA, whereas PSA expression is not prostate cancer specific.

PSCA is Expressed by a Subset of Basal Cells in Normal Prostate: Normal prostate contains two major epithelial cell populations--secretory luminal cells and subjacent basal cells. In situ hybridizations were performed on multiple sections of normal prostate using an antisense riboprobe specific for PSCA to localize its expression. As shown in FIG. 11, PSCA is expressed exclusively in a subset of normal basal cells. Little to no staining is seen in stroma, secretory cells or infiltrating lymphocytes. Hybridization with sense PSCA riboprobes showed no background staining. Hybridization with an antisense probe for GAPDH confirmed that the RNA in all cell types was intact. Because basal cells represent the putative progenitor cells for the terminally differentiated secretory cells, these results suggest that PSCA may be a prostate-specific stem/progenitor cell marker (Bonkhoff et al., 1994, Prostate 24: 114-118). In addition, since basal cells are androgen-independent, the association of PSCA with basal cells raises the possibility that PSCA may play a role in androgen-independent prostate cancer progression.

PSCA is Overexpressed in Prostate Cancer Cells: The initial analysis comparing PSCA expression in normal prostate and LAPC-4 xenograft tumors suggested that PSCA was overexpressed in prostate cancer. As demonstrated by the Northern blot analysis as shown in FIG. 9, LAPC-4 prostate cancer tumors strongly express PSCA; however, there is almost no detectable expression in sample of BPH. In contrast, PSA expression is clearly detectable in normal prostate, at levels 2-3 times those seen in the LAPC-4 tumors. Thus, the expression of PSCA in prostate cancer

appears to be the reverse of what is seen with PSA. While PSA is expressed more strongly in normal than malignant prostate tissue, PSCA is expressed more highly in prostate cancer.

To confirm the PSCA expression and its value in diagnosing prostate cancer, one hundred twenty six paraffin-embedded prostate cancer specimens were analyzed by mRNA in situ hybridization for PSCA expression. Specimens were obtained from primary tumors removed by radical prostatectomy or transurethral resection in all cases except one. All specimens were probed with both a sense and antisense construct in order to control for background staining. Slides were assigned a composite score as describe under Materials and Methods, with a score of 6 to 9 indicating strong expression and a score of 4 meaning moderate expression. 102/126 (81%) of cancers stained strongly for PSCA, while another 9/126 (7%) displayed moderate staining (FIGS. 11B and 11C). High grade prostatic intraepithelial neoplasia, the putative precursor lesion of invasive prostate cancer, stained strongly positive for PSCA in 82% (97/118) of specimens (FIG. 11B) (Yang et al., 1997, Am. J. Path. 150: 693-703). Normal glands stained consistently weaker than malignant glands (FIG. 11B). Nine specimens were obtained from patients treated prior to surgery with hormone ablation therapy. Seven of nine (78%) of these residual presumably androgen-independent cancers overexpressed PSCA, a percentage similar to that seen in untreated cancers. Because such a large percentage of specimens expressed PSCA mRNA, no statistical correlation could be made between PSCA expression and pathological features such as tumor stage and grade. These results suggest that PSCA mRNA overexpression is a common feature of androgen-dependent and independent prostate cancer.

PSCA is Expressed in Androgen Independent Prostate Cancer Cell Lines: Although PSCA was initially cloned using subtractive hybridization, Northern blot analysis demonstrated strong PSCA expression in both androgen-dependent and androgen-independent LAPC-4 xenograft tumors (FIG. 9). Moreover, PSCA expression was detected in all prostate cancer xenografts, including the LAPC-4 and LAPC-9 xenografts.

PSCA expression in the androgen-independent, androgen receptor-negative prostate cancer cell lines PC3 and DU145 was also detected by reverse-transcriptase polymerase chain reaction

analysis. These data suggest that PSCA can be expressed in the absence of functional androgen receptor.

Example 2: Biochemical Characterization Of PSCA

This experiment shows that PSCA is a glycosylated, GPI-anchored cell surface protein

MATERIALS AND METHODS

10 Polyclonal Antibodies and Immunoprecipitations: Rabbit polyclonal antiserum was generated against the synthetic peptide -TARIRAVGLLTVISK- and affinity purified using a PSCA-glutathione S transferase fusion protein. 293T cells were transiently transfected with pCDNA II (Invitrogen, San Diego, CA) expression vectors containing PSCA, CD59, E25 or vector alone by calcium phosphate precipitation. Immunoprecipitation was performed as previously described
15 (Harlow and Lane, 1988, Antibodies: A Laboratory Manual. (Cold Spring Harbor Press)). Briefly, cells were labeled with 500uCi of trans³⁵S label (ICN, Irvine, CA) for six hours. Cell lysates and conditioned media were incubated with 1ug of purified rabbit anti-PSCA antibody and 20ul protein A sepharose CL-4B (Pharmacia Biotech, Sweden) for two hours. For deglycosylation, immunoprecipitates were treated overnight at 37° with 1u N-glycosidase F (Boehringer Mannheim)
20 or 0.1 u neuraminidase (Sigma, St. Louis, MO) for 1 hour followed by overnight in 2.5 mU O-glycosidase (Boehringer Mannheim).

Flow Cytometry: For flow cytometric analysis of PSCA cell surface expression, single cell suspensions were stained with 2ug/ml of purified anti-PSCA antibody and a 1:500 dilution of
25 fluorescein isothiocyanate (FITC) labeled anti-rabbit IgG (Jackson Laboratories, West Grove, PA). Data was acquired on a FACScan (Becton Dickinson) and analyzed using LYSIS II software. Control samples were stained with secondary antibody alone. Glycosylphosphatidyl inositol linkage was analyzed by digestion of 2×10^6 cells with 0.5 units of phosphatidylinositol-specific phospholipase C (PI-PLC, Boehringer Mannheim) for 90 min at 37° C. Cells were analyzed prior
30 to and after digestion by either FACS scanning or immunoblotting.

RESULTS

PSCA is a GPI-Anchored Glycoprotein Expressed on the Cell Surface: The deduced PSCA amino acid sequence predicts that PSCA is heavily glycosylated and anchored to the cell surface through a GPI mechanism. In order to test these predictions, we produced an affinity purified polyclonal antibody raised against a unique PSCA peptide (see Materials and Methods). This peptide contains no glycosylation sites and was predicted, based on comparison to the three dimensional structure of CD59 (another GPI-anchored PSCA homologue), to lie in an exposed portion of the mature protein (Kiefer et al., 1994, Biochem. 33: 4471-4482). Recognition of PSCA by the affinity-purified antibody was demonstrated by immunoblot and immunoprecipitation analysis of extracts of 293T cells transfected with PSCA and a GST-PSCA fusion protein. The polyclonal antibody immunoprecipitates predominantly a 24kd band from PSCA-transfected, but not mock-transfected cells (FIG. 12A). Three smaller bands are also present, the smallest being ~10kd. The immunoprecipitate was treated with N and O specific glycosidases in order to determine if these bands represented glycosylated forms of PSCA. N-glycosidase F deglycosylated PSCA, whereas O-glycosidase had no effect (FIG. 12A). Some GPI-anchored proteins are known to have both membrane-bound and secreted forms (Fritz and Lowe, 1996, Am. J. Physiol. 270: G176-G183). FIG. 12B indicates that some PSCA is secreted in the 293T-overexpressing system. The secreted form of PSCA migrates at a lower molecular weight than the cell surface-associated form, perhaps reflecting the absence of the covalent GPI-linkage. This result may reflect the high level of expression in the 293T cell line and needs to be confirmed in prostate cancer cell lines and *in vivo*.

Fluorescence activated cell sorting (FACS) analysis was used to localize PSCA expression to the cell surface. Nonpermeabilized mock-transfected 293T cells, PSCA-expressing 293T cells and LAPC-4 cells were stained with affinity purified antibody or secondary antibody alone. FIG. 12C shows cell surface expression of PSCA in PSCA-transfected 293T and LAPC-4 cells, but not in mock-transfected cells. To confirm that this cell surface expression is mediated by a covalent GPI-linkage, cells were treated with GPI-specific phospholipase C (PLC). Release of PSCA from the cell surface by PLC was indicated by a greater than one log reduction in fluorescence intensity.

Recovery of PSCA in post digest conditioned medium was also confirmed by immunoblotting. The specificity of phospholipase C digestion for GPI-anchored proteins was confirmed by performing the same experiment on 293T cells transfected with the GPI-linked antigen CD59 or the non-GPI linked transmembrane protein E25a (Deleersnijder et al., 1996, J. Biol. Chem 271: 19475-19482). PLC digestion reduced cell surface expression of CD59 to the same degree as PSCA but had no effect on E25. These results support the prediction that PSCA is a glycosylated, GPI-anchored cell surface protein.

Example 3: Isolation Of cDNA Encoding Murine PSCA Homologue

The human PSCA cDNA was used to search murine EST databases in order to identify homologues for potential transgenic and knockout experiments. One EST obtained from fetal mouse and another from neonatal kidney were 70% identical to the human cDNA at both the nucleotide and amino acid levels. The homology between the mouse clones and human PSCA included regions of divergence between human PSCA and its GPI-anchored homologues, indicating that these clones likely represented the mouse homologue of PSCA. Alignment of these ESTs and 5' extension using RACE-PCR provided the entire coding sequence (FIG. 2).

Example 4: Isolation Of Human And Murine PSCA Genes

This experiment shows that PSCA is located at chromosome 8, band q24.2.

MATERIALS AND METHODS

Genomic Cloning: Lambda phage clones containing the human PSCA gene were obtained by screening a human genomic library (Stratagene) with a human PSCA cDNA probe (Sambrook et al., 1989, Molecular Cloning (Cold Spring Harbor)). BAC (bacterial artificial chromosome) clones containing the murine PSCA gene were obtained by screening a murine BAC library (Genome Systems, Inc., St. Louis, MO) with a murine PSCA cDNA probe. A 14kb human Not I fragment

and a 10kb murine Eco RI fragment were subcloned into pBluescript (Stratagene), sequenced, and restriction mapped.

5 Chromosome Mapping by Fluorescence In Situ Hybridization: Fluorescence in situ chromosomal analysis (FISH) was performed as previously described using overlapping human lambda phage clones (Rowley et al., 1990, PNAS USA 87: 9358-9362, H. Shizuya, PNAS USA, 89:8794).

RESULTS

10 Structure of PSCA Gene: Human and murine genomic clones of approximately 14kb and 10kb, respectively, were obtained and restriction mapped. A schematic representation of the gene structures of human and murine PSCA and Ly-6/Thy-1 is shown in FIG. 8. Both the human and murine genomic clones contain three exons encoding the translated and 3' untranslated regions of the PSCA gene. A fourth exon encoding a 5' untranslated region is presumed to exist based on
15 PSCA's homology to other members of the Ly-6 and Thy-1 gene families (FIG. 8).

Human PSCA Gene Maps to Chromosome 8q24.2: Southern blot analysis of LAPC-4 genomic DNA revealed that PSCA is encoded by a single copy gene. Other Ly-6 gene family members contain four exons, including a first exon encoding a 5' untranslated region and three additional
20 exons encoding the translated and 3' untranslated regions. Genomic clones of human and murine PSCA containing all but the presumed 5' first exon were obtained by screening lambda phage libraries. Mouse and human PSCA clones had a similar genomic organization. The human clone was used to localize PSCA by fluorescence in situ hybridization analysis. Cohybridization of overlapping human PSCA lambda phage clones resulted in specific labeling only of chromosome 8
25 (FIG. 13). Ninety seven percent of detected signals localized to chromosome 8q24, of which 87% were specific for chromosome 8q24.2. These results show that PSCA is located at chromosome 8, band q24.2.

30 Example 5: Generation Of Monoclonal Antibodies Recognizing Different Epitopes Of PSCA

Materials and Methods

Generation and Production of Monoclonal Antibodies. BALB/c mice were immunized three times with a purified PSCA-glutathione S-transferase (GST) fusion protein containing PSCA amino acids 22-99 (FIG. 1B). Briefly, the PSCA coding sequence corresponding to amino acids 18 through 98 of the human PSCA amino acid sequence was PCR-amplified using the primer pair:

5'- GGAGAATTCATGGCACTGCCCTGCTGTGCTAC
3'-GGAGAATTCCTAATGGGCCCCGCTGGCGTT

10

The amplified PSCA sequence was cloned into pGEX-2T (Pharmacia), used to transform *E. coli*, and the fusion protein isolated.

15 Spleen cells were fused with HL-1 myeloma cells using standard hybridoma technique. Hybridomas that were positive for PSCA by ELISA and FACS analysis (see Results) were subcloned. Ascites fluid was produced in C.B. 17 scid/scid mice and monoclonal antibodies (mAbs) purified using a protein G affinity column (Pharmacia Biotech, Piscataway, N.J.). PSCA mAb 1G8 was also produced in Cell-Pharm System 100 as recommended by the
20 manufacturer (Unisyn Technologies, Hopkinton, MA).

ELISA for Hybridoma Screening. GST or PSCA-GST were immobilized on Reacti-Bind maleic anhydride-activated polystyrene plates (Pierce, Rockford, IL). 50ul of hybridoma media were added to each well and incubated for 1 hour at room temperature. Wells were washed 3
25 times with 200ul PBS containing 0.1% BSA and 0.05% Tween 20 and incubated for 1 hour with 100ul anti-mouse IgG (1:4000) labeled with alkaline phosphatase (Promega, Madison, WI). Plates were developed with an alkaline phosphatase substrate (Bio-Rad, Hercules, CA).

Cell Culture. LNCaP was obtained from ATCC and stably transfected with a pCDNA II (Invitrogen) expression vector containing PSCA or vector alone (Reiter, R. et al., 1998). 293T cells transiently transfected with PSCA or vector alone were prepared as described previously (Reiter, R. et al., 1998). LAPC-9 xenograft explants were propagated in PrEGM media (Clonetics, San Diego, CA) after digestion in 1% pronase for 18 min. at room temperature. Before FACS analysis, LAPC-9 cells were passed through a 40um cell strainer to obtain single cell suspensions.

Immunofluorescence. Cells were grown on glass coverslips coated with poly-L-lysine.

- 10 Immunofluorescence assays were carried out on permeabilized and nonpermeabilized fixed cells. For fixation, cells were treated with 2% paraformaldehyde in PBS-CM (PBS, 100uM CaCl₂, 1mM MgCl₂) for 30 minutes in the dark, quenched with 50uM NH₄Cl in PBS-CM-BSA (PBS, 100uM CaCl₂, 1mM MgCl₂, 0.2% BSA) for 10 minutes, and washed twice with PBS-CM-BSA. For permeabilization, cells were treated additionally with PBS-CM-BSA-Saponin (0.075% saponin (Sigma) in PBS-CM-BSA) for 15 minutes at room temperature. Primary mAb at 2-5 mg/ml in PBS-CM-BSA (plus saponin in cases of permeabilization) was added for 60 minutes and washed twice with PBS-CM-BSA. FITC-conjugated goat antimouse IgG antibody (1:500 diluted in PBS-CM-BSA +/- saponin; Southern Biotechnology, Birmingham, AL) was added for 30 minutes and washed 3 times with PBS-CM. Slides were mounted in vectashield (Vector Laboratory, Inc., Burlingame, CA).

- Flow Cytometry.** Cells (1×10^6) were incubated for 30 minutes at 4°C with 100 ul mAb at 2ug/ml in PBS containing 2% fetal bovine serum or hybridoma conditioned medium. After washing, cells were stained with a 1:500 dilution of FITC-conjugated goat antimouse IgG (Southern Biotechnology, Birmingham, AL). Data was acquired on a FACScan (Becton Dickinson) and analyzed by using LYSIS II software.

Immunoblotting and Immunoprecipitation. Immunoprecipitation was performed as described (Harlow, E. and Lane, D., 1988). Briefly, cells were labeled with 500uCi of trans³⁵ label (ICN) for

6 hours. Cell lysates were incubated with 3ug mAb and 20ul of protein A-Sepharose CL-4B (Pharmacia Biotech) for 2 hours. For immunoblotting, protein extracts were prepared by lysing cells in 1X SDS Laemmli sample buffer and boiling for 5 min. Proteins were separated on 12.5% SDS polyacrylamide gels and transferred to nitrocellulose membranes, washed and incubated with 2ug mAb in 10ml blocking buffer (5% nonfat milk in TBST). Blots were developed using the Amersham enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL).

Immunohistochemistry. Normal formalin-fixed, paraffin-embedded tissue samples were obtained from the Departments of Pathology at Beth-Israel Deaconess Medical Center-Harvard Medical School and UCLA. Primary radical prostatectomy specimens were selected from a previously described database (Magi-Galluzzi, C. et al., 1997). Bone metastases and matched primary biopsy specimens were obtained from the UCLA Department of Pathology. Normal tissues were stained and scored independently at two institutions in order to ensure reproducibility. Specimens obtained from UCLA were stained using modifications of an immunoperoxidase technique previously described (Said, J. W. et al., 1998). Antigen retrieval was performed on paraffin sections using a commercial steamer and 0.01M citrate buffer pH 6.0. After incubation with PSCA mAbs for 50 min. (see below), slides were treated sequentially with rabbit anti-mouse IgG, swine anti-rabbit IgG and rabbit anti-swine IgG, all biotin conjugated. Slides were then incubated with streptavidin-peroxidase and antibody localization performed using the diaminobenzidine reaction. Specimens obtained from Beth-Israel-Deaconess-Harvard Medical School were stained as previously described using an automated Ventana NexES instrument (Ventana Medical Systems, Tucson, AZ) (Magi-Galluzzi, C. et al., 1997). Antigen retrieval was done by microwave for 15 min. in EDTA, pH 8.05 at 750W. mAbs purified at a concentration of ~ 1ug/ul from SCID ascites were used at the following concentrations: 1G8 = 1:20; 3E6 = 1:30; 2H9 = 1:50; 4A10 = 1:100; 3C5 = 1: 100. mAb 1G8 was produced in CellPharm System 100 and used at a concentration of 1:10. Positive controls included LAPC-9 and LNCaP-PSCA and negative controls were LNCaP and isotype-matched irrelevant antibody. Primary biopsy specimens were available for three patients with bone metastases. To approximate conditions of decalcification, slides from these specimens were treated for 20 min. in Decal-Stat (Lengers, NY) prior to staining with PSCA mAbs.

Monoclonal antibodies (mAbs) were raised against a PSCA-GST fusion protein lacking both the amino and carboxyl terminal signal sequences of PSCA. Positive fusions were selected by ELISA using the PSCA-GST fusion protein and GST alone. Out of 400 hybridomas screened, 28 recognized the PSCA-GST fusion but not GST alone. These fusions were screened

5 secondarily by flow cytometry of nonpermeabilized 293T cells transfected with PSCA and mock transfected 293T cells. Secondary screening by FACS was done in order to select clones capable of recognizing PSCA on the cell surface, hypothesizing that these might later become useful for *in vivo* targeting applications. Seven positive fusions were identified in this manner (mAbs 2A2, 3G3, 4A10, 1G8, 3E6, 3C5 and 2H9), of which five (mAbs 4A10, 1G8, 3E6, 3C5 and 2H9)
10 were subcloned and purified.

The mAbs were tested for their ability to immunoprecipitate PSCA and/or to recognize PSCA on immunoblots. All mAbs were able to immunoprecipitate PSCA from 293T-PSCA cells, as well as from LAPC-9 prostate cancer xenograft tumors that express high levels of endogenous
15 PSCA (Figure 37). Likewise, all mAbs detected PSCA by immunoblotting, although mAbs 2H9 and 3E6 recognized only the ~ 12 kd deglycosylated form of PSCA (Figure 34).

The location on PSCA of the epitopes recognized by the five mAbs was determined by immunoblot analysis using three truncated PSCA-GST fusion proteins. mAbs 4A10, 2H9 and
20 3C5 recognize an epitope residing within the amino-terminal portion of PSCA (i.e., amino acids 21-50); mAb 1G8 recognizes an epitope within the middle region of PSCA (i.e., amino acids 46-85); and mAb 3E6 reacts within the carboxyl-terminal portion of PSCA (amino acids 85-99) (Figure 15). All five mAbs are IgG as described in Figure 15. These results demonstrate that the five mAbs can detect PSCA in multiple assays and recognize at least three distinct epitopes
25 on PSCA.

PSCA mAbs Stain the Cell Surface of Prostate Cancer Cells

The utility of mAbs for studying PSCA biology and for potential clinical applications such as *in*

vivo targeting applications is dependent on their ability to recognize the antigen of interest on the plasma membrane (Liu, H. et al., 1997; McLaughlin, P. et al., 1998; Wu, Y. et al., 1995; Tokuda, Y. et al., 1996). In order to determine the ability of mAbs 2H9, 3E6, 1G8, 4A10 and C5 to recognize PSCA specifically on the cell surface of prostate cancer cells, LNCaP cells transfected with PSCA (LNCaP-PSCA) and LAPC-9 cells were examined by flow cytometry and indirect immunofluorescence. As with 293T-PSCA cells, all five mAbs were able to detect PSCA on the cell surface of nonpermeabilized LNCaP-PSCA and/or LAPC-9 cells by flow cytometry (Figure 33). Mock-transfected LNCaP and LNCaP transfected with a neomycin-alone containing vector (LNCaP-neo), neither of which expresses detectable PSCA mRNA, were both negative.

Immunofluorescent analysis was performed on both permeabilized and nonpermeabilized cells in order to ascertain whether PSCA protein localizes to the cell surface (Liu, H. et al., 1997). Nonpermeabilized LNCaP-PSCA showed clear cell surface reactivity with mAbs 1G8, 3E6, 4A10 and 3C5, but did not stain with mAb 2H9 (mAb 2H9 also did not detect PSCA on LNCaP-PSCA cells by FACS). LAPC-9 cells showed cell surface reactivity with all five mAbs (Figure 35). LNCaP-neo, as predicted, was negative both with and without permeabilization. Permeabilization of LNCaP-PSCA and LAPC-9 resulted in both membrane and cytoplasmic staining. All mAbs produced a punctate staining pattern on the cell surface, which was most pronounced with mAbs 3E6, 3C5 and 4A10 (Figure 35). This pattern may reflect aggregation or clustering of PSCA to regions of the cell surface. These results demonstrate that all five mAbs react with PSCA on the cell surface of intact prostate cancer cells.

Immunohistochemical Staining of PSCA in Normal Prostate

PSCA mRNA localizes to a subset of basal cells in normal prostate, suggesting that PSCA may be a cell surface marker for prostate stem/progenitor cells (Reiter, R. et al., 1998). In order to test the possibility that PSCA protein may be a marker of basal cells, PSCA expression was examined immunohistochemically in paraffin-embedded sections of normal prostate. mAbs 1G8

and 2H9 stained the cytoplasm of both basal and secretory cells , while mAb 3E6 reacted predominantly with basal cells (Figure 38). Atrophic glands, which express basal cell cytokeratins, stained strongly with all three mAbs (Figure 38) (O'Malley, F. P. et al., 1990). mAbs 3C5 and 4A10 gave strong background staining and/or nonspecific nuclear staining in paraffin sections and were not used further. These results suggest that although PSCA mRNA is detected specifically in basal cells, PSCA protein can be detected in both epithelial cell layers (i.e. basal and secretory) of the prostate, although there are some differences in the staining patterns of individual antibodies.

10 Immunohistochemical Analysis of Normal Tissues

Our initial studies indicated that PSCA expression in men was largely prostate-specific, with low levels of detectable RNA in kidney and small intestine. PSCA mRNA was also detected in placenta. The prostate-specificity of PSCA protein expression was tested by immunohistochemical staining of 20 tissues using mAb 1G8 (see Table 1). Positive tissue staining with mAb 1G8 was confirmed with mAbs 2H9 and/or 3E6 in order to ensure reproducibility with mAbs directed against distinct epitopes. Staining was also performed and scored independently at two institutions in order to confirm the results. As predicted by the RNA analysis, placenta was positive with all mAbs tested, with cytoplasmic staining detected in the trophoblasts (Figure 39A). In kidney staining was detected in the collecting ducts and distal convoluted tubules, but not in glomeruli (Figure 39A). Transitional epithelium of the bladder and ureter, which had not been examined previously at the mRNA level, was positive with all mAbs tested (Figure 39A). The only other tissue with significant immunoreactivity was colon, in which single cells deep within the crypts stained intensely positive (Figure 39A). Double staining with chromogranin indicated that these cells are of neuroendocrine origin.

In order to confirm that mAb reactivity in bladder represented PSCA, Northern blot analysis was performed on three normal bladder samples obtained at radical cystectomy and compared with PSCA expression in prostate, kidney and the LAPC-9 xenograft (Figure 39B). PSCA

mRNA was detected in bladder at levels lower than those seen in prostate, confirming the immunohistochemical result. No signal was detected in the three kidney specimens, consistent with our previous observation that PSCA expression in kidney is significantly lower than prostate (Reiter, R. et al., 1998). LAPC-9, a prostate cancer xenograft established from a bone metastasis, expresses very high levels of PSCA mRNA compared with normal bladder and prostate (Whang, Y. E. et al., 1998). These results confirm that PSCA expression in men is largely prostate-predominant; however, there is also detectable PSCA protein expression in urothelium, renal collecting ducts and colonic neuroendocrine cells.

10 PSCA Protein is Expressed by a Majority of Localized Prostate Cancers

In our previous study, mRNA was expressed in ~ 80% of tumors and appeared to be expressed more highly in normal than malignant glands (Reiter, R. et al., 1998). In order to determine if PSCA protein can be detected in prostate cancers and if PSCA protein levels are increased in malignant compared with benign glands, paraffin-embedded pathological specimens of primary and metastatic prostate cancers were immunostained with mAb 1G8 (Figures 21 and 28). Isolated cases were also stained with mAbs 3E6 or 2H9 in order to confirm the specificity of the staining. Twelve of 15 primary cancers stained positive (Figure 21), including 2/2 cases containing foci of high grade prostatic intraepithelial neoplasia. Staining intensity varied, with 7 cases showing equivalent staining in cancer and adjacent normal glands and 5 showing significantly stronger staining in cancer. In some cases there was strong expression in the malignant glands and undetectable staining in adjacent normal tissue (Figure 21; patient 1). Also, there were some cases in which staining was heterogeneous, with some malignant glands staining more strongly than others (Figure 21; patient 2). Overall, poorly differentiated tumors stained more strongly than well differentiated ones, suggesting that PSCA overexpression may correlate with increasing tumor grade (Figure 21; patient 3). These results demonstrate that PSCA protein is expressed in prostate cancer. Consistent with our previous mRNA in situ studies, PSCA appears to be overexpressed in a significant percentage of cancers, perhaps in concert with increasing tumor grade.

This study describes the first characterization of PSCA protein expression using five monoclonal antibodies directed against PSCA. Because these mAbs recognize epitopes on the exterior of the cell surface, they may have utility for prostate cancer diagnosis and therapy (Liu, H. et al., 1997). One possibility is that these mAbs could be used to locate sites of metastatic disease, similar to the Prostatecint scan which uses an antibody directed against PSMA (Sodee, D. B. et al., 1996). Another possibility is that they may be used to target prostate cancer cells therapeutically, either alone or conjugated to a radioisotope or other toxin. Similar approaches are currently being evaluated using antibodies directed against extracellular epitopes on PSMA (Murphy, G. P. et al., 1998; Liu, H. et al., 1997; Liu, H. et al., 1998).

PSCA mAbs stain the cell surface in a punctate manner, suggesting that PSCA may be localized to specific regions of the cell surface. GPI-anchored proteins are known to cluster in detergent-insoluble glycolipid-enriched microdomains (DIGS) of the cell surface (Varma, R. and Mayor, S., 1998). These microdomains, which include caveolae and sphingolipid-cholesterol rafts, are believed to play critical roles in signal transduction and molecular transport (Anderson, R. Caveolae, 1993; Friedrichson, T. and Kurzchalia, T. V., 1998; Hoessli, D. C. and Robinson, P. J., 1998). Thy-1, a homologue of PSCA, has previously been shown to transmit signals to src kinases through interaction in lipid-microdomains (Thomas, P. M. and Samuelson, L. E., 1992; Stefanova, I. et al., 1991). Preliminary subcellular fractionation experiments in our laboratory confirm the presence of PSCA in DIGS (Xavier, R. et al., 1998).

GPI-anchored proteins have also been reported to localize to prostasomes, membrane-bound storage vesicles released by prostate epithelial cells (Ronquist, G. and Brody, I., 1985). CD59, a GPI-anchored inhibitor of complement-mediated cytolysis, is found in high concentrations in prostasomes of normal prostate epithelial cells and prostatic secretions (Rooney, I. et al., 1993). PSCA protein is detected in prostate secretory cells.

Contrary to our previous finding that PSCA mRNA localized exclusively to basal cells, the current results suggest that PSCA protein may be present in both basal and secretory cells.

Similar differences between mRNA and protein localization in prostate have been described for PSMA and androgen receptor (Magi-Galuzzi, C. et al., 1997; Kawakami, M. and Nakayama, J., 1997). One possible explanation for the presence of PSCA protein in secretory cells is that PSCA mRNA is transcribed in basal progenitor cells but that PSCA protein expression persists as basal cells differentiate into secretory cells. Another possibility is that PSCA protein may be transferred from basal to secretory cells posttranslationally.

- 10 Differences in staining intensity of basal and secretory cells by mAbs 3E6, 1G8 and 2H9 may reflect the distinct epitopes recognized by the antibodies and/or differences in posttranslational modification of PSCA in basal and secretory cells. Supporting this possibility is the observation that the five mAbs do not react equally with PSCA in all assays or cell lines. mAb 2H9 recognizes PSCA on the cell surface of LAPC-9 but not LNCaP-PSCA, suggesting that the
- 15 epitope recognized by this antibody may be altered or obscured in the latter cell type. We have also observed that mAb 3E6 does not stain cancers as strongly as mAbs 1G8 and 2H9 in some cases, suggesting that it may react with certain forms of PSCA preferentially.

- Although largely prostate-specific in men, PSCA is also expressed at lower levels in urothelium,
- 20 colonic neuroendocrine cells, and renal tubules and collecting ducts. The staining seen in renal tubules and collecting ducts is interesting in that these structures derive embryologically from the ureteric bud of the mesonephric duct, suggesting a possible reason for the staining patterns seen in kidney. The absence of detectable PSCA mRNA in kidney specimens may reflect either low levels of expression or the possibility that the samples were obtained primarily from the
- 25 renal cortex, whereas the collecting ducts are located in the renal medulla.

The primary impetus for identifying prostate-specific cell surface genes is the desire to develop selective, nontoxic therapies. PSMA, another "prostate-restricted" protein, has also been shown to be expressed in duodenum, colonic neuroendocrine cells and proximal renal tubules (Silver,

D. A. et al., 1997). Preliminary reports of PSMA vaccine therapy have not produced significant toxicity (Tjoa, B. A. et al., 1998).

Expression of PSCA in urothelium and kidney appears to be lower than in normal prostate and significantly less than that seen in many of the prostate cancers evaluated. Therapies directed against PSCA may therefore be relatively selective for cancer, much as Her-2/neu antibodies primarily target breast cancers that overexpress Her-2/neu (Disis, M. L. and Cheever, M. A., 1997).

Expression of PSCA in urothelium and kidney raises the possibility that it may be expressed in transitional and renal cell carcinomas. Two bladder cancers examined do express PSCA, one at levels similar to LAPC-9, suggesting that PSCA may be overexpressed in some cases of transitional cell carcinoma. A more complete survey of bladder cancer specimens will be required to test this possibility.

The data herein supports our earlier observation that PSCA is expressed in a majority of prostate cancers. Likewise, PSCA protein is overexpressed in some prostate tumors when compared to adjacent normal glands, supporting its use as a target for prostate cancer therapy. In contrast to the mRNA in situ studies, the current results suggest that PSCA protein expression may correlate with cancer stage and/or grade. Similar differences between RNA and protein expression have been noted for thymosin Beta-15 (Bao, L., et al., 1996).

Table 1. PSCA expression in normal tissues.

<u>Staining</u>	<u>Tissue</u>
Positive	Prostate (epithelium) Bladder (transitional epithelium) Placenta (trophoblasts) Colon (neuroendocrine cells) Kidney (tubules and collecting duct)*
Negative	Kidney (glomeruli)

5 Prostate (stroma)
Bladder (smooth muscle)
Testis
Endometrium
Small intestine
Liver
Pancreas
Breast
Gallbladder
10 Skeletal muscle
Brain
Peripheral nerve
Bone marrow
Thymus
15 Spleen
Lung
Bronchus
Heart

20 * mAb 3E6 reacts with the distal convoluted tubule, while mAb 1G8 reacts with distal tubules and, in some cases, proximal tubules.

Example 6

25 This experiment shows that PSCA expression is amplified in bone metastases of prostate cancer.

MATERIALS AND METHODS

30 Horse Serum (NHS) (GIBCO #26050-070) was diluted (1/20 dilution) in 1% Casein, PBST. The antibodies of the invention that recognize PSCA were diluted in 1/100 NHS, PBST.

The detection system included HRP-rabbit anti-mouse Ig (DAKO P260), HRP-swine anti-rabbit Ig (DAKO P217), HRP-rabbit anti-swine Ig (DAKO P164). Each were diluted 1/100 in 1/100 NHS, PBST.

35

3,3'-diaminobenzidine tetrahydrochloride (DAB) (Fluka) stock was made by dissolving 5 gm in 135 ml of 0.05 M Tris, pH 7.4. DAB was aliquoted into 1 ml/vial and frozen at -20°C . A working solution of DAB was made by adding 1 ml of DAB to 40 ml of DAB buffer and 40 microliters of 50% H_2O_2 .

5

DAB buffer was prepared by combining 1.36 gm Imidazole (Sigma #I-0125) with 100 ml $\text{D}^2\text{-H}_2\text{O}$, then adjusting the pH to 7.5 with 5 N.HCl. After the pH adjustment 20 ml of 0.5 M Tris pH 7.4 and 80 ml of $\text{D}^2\text{-H}_2\text{O}$ were added.

10 A section of a tissue/tumor known and previously demonstrated to be positive for the antibody was run with the patient slide. This slide served as a "positive control" for that antibody. A section of the patient's test specimen was incubated with a negative control antibody in place of the primary antibody. This slide served as a "negative control" for the test.

15 The staining procedure was as follows. Bone samples were applied to slides. The slides were then baked overnight at 60°C . Slides were deparaffinize in 4 changes of xylene for 5 minutes each and passed through a graded series of ethyl alcohol (100% x 4, 95% x 2) to tapwater then transferred to NBF, and fixed for 30 minutes. The fixed slides were placed in running tapwater for 15 minutes, transferred to 3% H_2O_2 -MeOH, incubated for 10 minutes, and washed in running tapwater for 5
20 minutes, then rinse in deionized water.

Slides were then subjected to 0.01 M citrate buffer pH 6.0, heated at 45°C for 25 minutes, cooled for 15 min and then washed in PBS. The slides were then rinsed in PBS and placed onto programmed DAKO Autostainer, using the following four step program. The four step
25 program is as follows. The slide is rinsed in PBS and blocked with 1/20 NHS in 1% Casein in PBST for 10 minutes. Primary antibody is then applied and incubated for 30 minutes followed by a buffer rinse. HRP-Rabbit anti-Mouse Ig is then applied and incubated for 15 minutes followed by another buffer rinse. HRP-Swine anti-Rabbit Ig is applied and incubated for 15

minutes followed by a buffer rinse. HRP-Rabbit anti-Swine Ig is applied and incubated for 15 minutes followed by a buffer rinse.

DAB is then applied to the slide and incubated for 5 minutes followed by a buffer rinse. A second
5 DAB is applied and incubated for 5 minutes followed by a buffer rinse.

The slides are removed from the Autostainer and placed into slide holders, rinsed in tapwater and counter stained with Harris hematoxylin (15 seconds). The slide is then washed in tapwater, dipped in acid-alcohol, washed in tapwater, dipped in sodium bicarbonate solution, and washed in
10 tapwater. The slides are then dehydrated in graded ethyl alcohols (95% x 2, 100% x 3) and Propar x 3 and coverslipped with Permount.

PSCA Protein is Expressed Strongly in Prostate Cancers Metastatic to Bone

15 Prostate cancer is unique among human tumors in its propensity to metastasize preferentially to bone and to induce osteoblastic responses. Nine sections of prostate cancer bone metastases were examined immunohistochemically (Figure 28). All reacted intensely and uniformly with mAb 1G8 (and/or 3E6). In two instances, micrometastases not readily detectable on hematoxylin and eosin sections could be seen after staining with mAb 1G8 (Figure 28; patient
20 5). Overall, staining in bone metastases was stronger and more uniform than in the primary tumors. In three cases, biopsy specimens from the primary tumors were available for comparison. All were weakly positive for PSCA when compared with the matched bone metastasis, suggesting that PSCA expression was increased in bone. In one biopsy specimen, weak staining was present in only a small focus of malignant glands, while the remaining tumor
25 was negative (Figures 21 and 28; Patient 4). In two cases, the biopsy specimens were obtained 10 and 15 years prior to the bone metastasis, indicative of a long latency period between the development of the primary and metastatic lesions. To rule out the possibility that the strong staining in bone was caused by the decalcification process used to prepare bone sections, the three primary biopsy specimens were also treated with decalcification buffer. Although this

treatment increased background staining, it did not alter epithelial reactivity significantly, indicating that the strong signal in bone was unlikely to be caused by the decalcification process. These results suggest that PSCA may be selected for or upregulated in prostate cancer metastases to bone.

5 FIGS. 21-23 show the bone samples of bone metastases of prostate cancer were positive for PSCA. Nine sections of prostate cancer bone metastases were examined. Consistent, intense staining was seen in nine prostate cancer bone metastases and all reacted intensely and uniformly with mAb 1G8 (and/or 3E6). In two instances, the pathologist could not readily identify the metastasis until staining with 1G8
10 highlighted the lesion. Overall, staining in bone metastases appeared stronger and more uniform than in the primary tumors.

These results suggest that PSCA may be greatly overexpressed in prostate cancer metastases to bone. This is particularly interesting since Sca-2, a close homologue of PSCA, was recently reported to
15 suppress osteoclast activity in bone marrow. If PSCA had similar activity, it might provide one explanation for the tendency of prostate cancer metastases to produce an osteoblastic response, since inhibition of osteoclast activity would tilt the balance of activity in bone to bone formation. Another possibility is that PSCA might be involved in adhesion to bone, since other Ly-6/Thy-1 family members are involved in similar processes. There was heterogeneous expression of PSCA in a number of
20 primary prostate cancers. These results further support the use of PSCA as a novel target for advanced disease.

One of the most intriguing results of the present study was the consistent, intense staining seen in the nine prostate cancer bone metastases. LAPC-9, a xenograft established from a bony
25 metastasis, also stained intensely for PSCA. In three patients, matched primary biopsy specimens showed low levels of PSCA expression compared to the bony metastases. Areas of strong PSCA expression in the primary tumors of the three patients examined may have been missed since only biopsies were available for analysis. Heterogeneous expression of PSCA was detected in at least one matched primary tumor, as well as in number of primary tumors for
30 whom matched metastatic lesions were not available. Also, in two cases the primary tumor was

sampled at least a decade prior to the bone metastasis, raising the possibility that clones expressing high levels of PSCA within the primary could have developed subsequent to the initial biopsy. These results clearly demonstrate PSCA expression in bone metastases, further supporting it as a novel target for advanced disease.

5

Example 7

This experiment shows that PSCA expression is higher in bladder carcinomas than normal bladder.

10

Tissues from prostate, bladder, kidney, testes, and small intestine (including prostate cancer and bladder and kidney carcinomas) were obtained from patients. These tissues were then examined for binding to PSCA using northern and western blot analyses as follows.

15 For northern blot analyses, tissue samples were excised and a less than 0.5 x 0.5 cm tissue sample was quick frozen in liquid nitrogen. The samples were homogenized in 7 mls of Ultraspec (Biotechx, Houston, Texas), using a polytron homogenizer using the protocol provided by Biotechx (UltraspecTM RNA Isolation System, Biotechx Bulletin No:27, 1992).

20 After quantification, 20 µg of purified RNA from each sample were loaded onto a 1% agarose formaldehyde gel. Running and blotting conditions were the same as was used in Example 1. The filters were separately probed with labeled PSCA and an internal control, actin. Filters were washed and exposed for several hours-overnight.

25 For western blot analyses, tissue samples were excised and a less than 0.5 x 0.5 cm tissue sample was taken and quickly minced and vortexed in equal volume of hot 2X Sample Buffer (5%SDS, 20% glycerol). Samples were incubated at 100° for 5 mins, vortexed and clarified for 30 min. Protein concentrations were determined by Biorad's DC Protein Assay kit (Richmond, CA). 40µg/sample was loaded on a 12% polyacrylamide protein gel. Transfer to a nitrocellulose filter

was done by standard methods (Towbin et al. PNAS 76:4350 (1979). A western blot was performed by incubating the filter with IG8 primary antibody followed by a secondary antibody, i.e., a goat α mouse IgG HRP. Detection was by Amersham ECL Detection kit (Arlington Heights, IL).

5

IG8 recognized and bound the PSCA on the cells surface of LAPC9 and a bladder carcinoma (designated bladder (Rob)) in a western blot analysis (FIG. 6). In FIG. 6, all tissues except LAPC9 were normal. A northern blot analysis confirmed elevated PSCA in the bladder carcinoma tissue (designated bladder (Rob) (also referred to as Rob's Kid CA) and LAPC9) (FIG. 25).

10

Example 8

This experiment shows that PSCA gene copy number is increased similar to an increase in copy number of c-myc (Figure 17). This is important because c-myc amplification correlates with poor outcome. Thus, the data suggests that PSCA amplification may also be a predictor for poor outcome.

15

FISH with Chromosome Enumeration Probes and a Probe for c-Myc.

20 The method of FISH is well known (Qian, J. et al., "Chromosomal Anomalies in Prostatic Intraepithelial Neoplasia and Carcinoma Detected by Fluorescence *in vivo* Hybridization," *Cancer Research*, 1995, 55:5408-5414.) Briefly, tissue sections (samples 34 and 75 were from two patients) were deparaffinized, dehydrated, incubated in 2X SSC at 75°C for 15 min, digested in pepsin solution [4mg/ml in 0.9% NaCl (pH 1.5)] for 15 min at 37°C, rinsed in 2X SSC at room temperature for 5 min, and air-dried.

25

Directly labeled fluorescent DNA probes for PSCA and for the 8q24 (c-myc) region were chosen. The PSCA cDNA (Fig. 1) was used to identify a 130 kb bacterial artificial chromosome (bac) clone (PSCA probe) that in turn was used in the FISH analysis in accordance with the

manufacturer's protocol (Genome Systems Inc.) The bac clone so identified and used in the FISH analysis was BACH-265B12 (Genome Systems, Inc. control number 17424).

5 Dual-probe hybridization was performed on the serial 5- μ m sections using a SG-labeled PSCA probe together with a SO-labeled probe for 8q24 (c-myc). Probes and target DNA were denatured simultaneously in an 80°C oven for 5 min. and each slide was incubated at 37°C overnight.

10 Posthybridization washes were performed in 1.5 M urea/0.1X SSC at 45°C for 30 min and in 2X SSC at room temperature for 2 min. Nuclei were counter-stained with 4,6-diamidino-2-phenylindole and anilfade compound *p*-phenylenediamine.

15 The number of FISH signals was counted with a Zeiss Axioplan microscope equipped with a triple-pass filter (I02-104-1010; VYSIS). The number of c-myc signals and PSCA signals were counted for each nucleus, and an overall mean c-myc:PSCA ratio was calculated. Results are shown in Figure 17.

20 The results show that PSCA gene copy number increased in prostate cancer samples (Figure 17). The PSCA gene is located at 8q24.2. The increase in gene copy number is due to both a gain in chromosome 8, and amplification of the PSCA gene (Figure 17). Interestingly, the increase in PSCA gene copy number is similar to an increase in gene copy number of c-myc (Figure 17) which is also located at 8q24. A previous study has demonstrated that a gain of chromosome 8 and amplification of c-myc are potential markers of prostate carcinoma progression (R B Jenkins *et al* 1997 *Cancer Research* 57: 524-531).

25 Example 9

Reporter gene construct using the hPSCA 9 kb upstream region to drive luciferase expression

The 14 kb NotI genomic fragment encoding the human PSCA gene was isolated from λ FIXII library encoding human genomic DNA (Stratagene), by screening the library with a full length human PSCA cDNA probe, as described in example 4 (Sambrook et al., 1989, Molecular Cloning (Cold Spring Harbor). The 14 kb human PSCA genomic fragment includes 9 kb of PSCA upstream sequences that was used to drive expression of a reporter gene.

The reporter gene vectors are depicted in Figure 42 and were constructed as follows. The 14 kb NotI fragment was sub-cloned from the λ vector into a Bluescript KS vector (Stratagene), resulting in the pBSKS-PSCA (14kb) construct. The PSCA upstream sequence was subcloned from pBSKS-PSCA(14 kb) by PCR amplification using a primer corresponding to the T7 sequence contained within the Bluescript vector, and a primer corresponding to a sequence contained within PSCA exon 1 (primer H3hPSCA3'-5, the sequence of this primer is as follows: The sequence of H3hPSCA3'-5 is 5'-gggaagcttgacagccttcagggtc-3'. The primer corresponding to PSCA exon 1 contained an introduced HindIII sequence to allow further subcloning following PCR amplification. The resulting amplified fragment was digested with HindIII and was subcloned into the pGL3-basic vector (Promega) to generate pGL3-PSCA (7 kb) which was used to generate a series of deletion reporter gene constructs containing varying lengths of PSCA upstream sequences operatively linked to the luciferase gene (Figure 42). The deleted portions of the PSCA upstream regions were obtained by subcloning restriction fragments from pGL3-PSCA (7 kb). The PSCA upstream region between -9 kb and -7 kb was subcloned from the pBSKS-PSCA (14 kb) construct, the NotI site was converted into a blunt end by Klenow and the fragment was cloned into the SacI/HindIII sites of pGL-PSCA (7 kb) in order to obtain the pGL3-PSCA (9 kb) construct. The reference to the sequences upstream of the PSCA coding region, such as -9 kb and -6 kb (etc.), are relative to the ATG start translation codon. The reporter gene constructs pGL3-PSCA (9 kb), pGL3-PSCA (6 kb), pGL3-PSCA (3 kb), and pGL3-PSCA (1 kb) were operatively linked to the luciferase gene (Figure 42). Plasmid, pGL3-CMV contains the cytomegalovirus promoter (Boshart, M. et al., 1985 *Cell* 41:521-530) linked to the luciferase gene and was used as a positive control. Also, plasmid pGL3 contains no promoter sequence and was used as a negative control plasmid.

Example 10

Transfection assay using a reporter gene construct containing the hPSCA upstream region.

- 5 Triplicate dishes of prostate and non-prostate cell lines were transfected by Tfx50 (Boeringer
Manheim) with the PSCA construct pGL3-PSCA (9 kb), or the positive control construct, pGL3-
CMV both described in Example 9 above, and assayed for luciferase activity (Figure 43). The
cells and cell lines transfected include PrEC (androgen-independent prostate basal cell), LNCaP
(androgen-dependent prostate secretory cell line), LAPC4 (androgen-dependent prostate cell line),
10 HT1376 (bladder cell line), and 293T (kidney cell line). Expression activities of the constructs are
expressed as a percentage of the activity of the CMV promoter. Standard errors are indicated
above the bars.

The results show that 9 kb of human PSCA upstream sequences drives expression of the
15 luciferase gene in a tissue-specific manner similar to the mRNA expression patterns seen for
native hPSCA shown in Figure 10 (Example 1). Luciferase was readily detectable in both
androgen-dependent and androgen-independent prostate cell lines and bladder. Luciferase was
also detectable, although at a lower level, in kidney cells.

Example 11

20 Identification of regulatory elements within the PSCA upstream region

Triplicate dishes of PrEC (Clonetech) or LNCaP cells were transfected with the reporter gene
constructs or the positive control construct described in Example 9 above, and assayed for
luciferase activity. The reporter gene constructs comprise various lengths of the hPSCA upstream
25 region operatively linked to the luciferase gene. The positive control construct, pGL3-CMV,
comprises the CMV promoter operatively linked to the luciferase. The cells were transfected
using a Tfx50 transfection system (Promega). Expression of luciferase in the transfected cells
were assayed using a Dual Luciferase Reporter Assay System (Promega), and the level of
luciferase expression was measure a relative luciferase unit (RLU).

30

The ability of the various lengths of the hPSCA upstream region to drive luciferase expression are expressed as a percentage of the activity of the positive control construct containing the CMV promoter. Standard errors are indicated.

- 5 The results shown in Figure 44 demonstrate that 3 kb of hPSCA upstream sequences drives expression of luciferase in both PrEc and LNCaP cells, but the level of detectable luciferase is 6 times higher in the LNCaP cells compared to the PrEC cells. This comparison was based on the level of detectable luciferase. In contrast, 1 kb of hPCSA upstream sequences did not drive expression of luciferase in either cell line.

10

Example 12

A Targeting vector

- A targeting vector was designed to delete the endogenous PSCA coding region, by homologous recombination. Figure 40 depicts a targeting vector for the mouse PSCA gene, and the strategy for using the targeting vector to delete the endogenous PSCA gene contained in a mouse cell. A targeting vector comprising a 12 kb SpeI fragment containing mouse PSCA upstream sequences, a NotI/EcoRI fragment containing the PGK promoter operatively linked to a neo^r gene from the pGT-N29 vector (New England BioLabs), and a 3.5 kb BstXI/XhoI fragment containing mouse PSCA downstream sequences. Constitutive expression of the neomycin resistance gene is controlled by the PGK promoter, and allows antibiotic selection of the targeted cells that contain the targeting vector.
- 15
- 20

- As understood by one skilled in the art, the targeting vector described here includes but is not limited to the neo^r gene for selection of the cells that contain the targeting vector or can contain no selectable reporter gene. The targeting vector can also be used to generate transgenic mice, known in the art as knock-in or knock-out mice, depending on whether the targeting vector contains a reporter gene or not, respectively. The transgenic mice can be used as an animal model to study the function of the PSCA gene in prostate development of mice.
- 25

As an example that is not intended to be limiting, the targeting vector was used to delete the wild type endogenous genomic mouse PSCA coding sequences in embryonic stem cells (ES) cells to generate cells that are heterozygous, containing a deleted PSCA gene. For example, the heterozygous cells generated using the targeting vector are PSCA⁺/neo^r as shown by the results in Figure 40. The phenotype of the heterozygous cells or transgenic mice can be compared with that of wild type PSCA cells or animals.

The targeting vector was constructed as follows. The ends of the 12 kb SpeI fragment containing the PSCA upstream and part of exon 1 sequences was blunt-ended and linked to the blunt-ended NotI/EcoRI fragment from pGT-N29 (BioLabs) containing the neomycin-resistance gene. The 3' end of the neomycin-resistance gene was linked to a blunt-ended 3.5 kb BstXI/XhoI fragment containing part of PSCA exon 3 and the downstream sequences. The resulting fragment was cloned into pGT-N29 to generate the targeting vector pGT-N29-mPSCA5'/3'.

The targeting vector was transfected into ES cells by electroporation using the method described in the following: Teratocarcinomas and Embryonic Stem Cells; A Practical Approach. IRL Press, Oxford (1987). Neomycin-resistant cells were selected and genomic DNA was isolated from the selected cells. A genomic Southern analysis was performed to determine the outcome of the homologous recombination reaction. 10 µg of DNA from the homologous recombination reaction and non-targeted ES cells were digested with EcoRI and analyzed by the Southern blot method (Southern, EM 1975 J. Molec. Biol. 98:503). The blot was probed with a XhoI/EcoRI fragment that contains sequences 3' to the PSCA coding region. The results show that the probe detects a 10 kb fragment that corresponds to the control non-targeted cells that are PSCA⁺/PSCA⁺, and a 4 kb fragment that corresponds to the targeted cells that are heterozygous and contain PSCA⁺/neo^r.

Example 13

Transgenic mouse models for prostate cancer

The present invention contemplates a strategy to generate transgenic mouse models for prostate cancer, using the upstream regions of the PSCA gene to drive expression of an oncogene, to induce tumor formation in prostate basal cells. As shown in Figure 41, the strategy involves administration, e.g., microinjection, of a chimeric oncogene vector, comprising the upstream
5 region of the PSCA gene operatively linked to a transgene that encodes a gene product that induces formation of a tumor. Other researchers have used this technique, using different prostate and non-prostate regulatory sequences operatively linked to an oncogene. For example, C3(1) is a prostate-predominant regulatory sequence (Moroulakou *et al* 1994 Proc. Nat. Acad. Sci. 91: 11236-11240) and probasin is a prostate-specific regulatory sequence (Greenberg *et al* 1995 Proc.
10 Nat. Acad. Sci. 92: 3439-3443), and both of these regulatory sequences drive expression of a transgene in prostate secretory cells. Cryptdin2 is a small-intestine predominant regulatory sequence (Garagenian *et al* Proc. Nat. Acad. Sci. 95: 15382-15387) that caused expression of an oncogene in prostate endocrine cells. In contrast, the present invention contemplates using the PSCA upstream region to drive expression of an oncogene in prostate basal cells, in order to
15 generate a transgenic mouse model for prostate cancer.

The clinical characteristics of the induced prostate tumor can be analyzed and compared with known characteristics of tumors caused by the particular oncogene used in constructing the chimeric oncogene vector. In addition, various tissues and organs of the transgenic mouse can be
20 analyzed by DNA, RNA and proteins analyses to ascertain the presence and expression patterns of the chimeric oncogene vector.

Example 14

Transgenic mice carrying chimeric vectors comprising hPSCA upstream sequences and a transgene

- 5 The expression patterns of transgenes under the control of hPSCA upstream regions will be tested. Toward this end, chimeric mice carrying chimeric vectors comprising hPSCA upstream sequences and a transgene have been generated. Chimeric vectors comprising 9 kb or 6 kb of hPSCA upstream sequences operatively linked to a transgene were constructed, and are schematically represented in Figure 45. The transgenes included green fluorescent protein cDNA (GFP, Clontech) linked to the SV40 polyadenylation sequence (PSCA (9 kb)-GFP and PSCA (6 kb)-GFP), green fluorescent protein cDNA linked to a 3' region of the human growth hormone that contains an intron cassette that confers stability to mRNA (PSCA (9 kb)-GFP-3'hGH and PSCA (6 kb)-GFP-3'hGH) (Brinster *et al* 1988 PNAS 85: 836-840), and the genomic fragment encoding SV40 small and large T antigen including an intron (PSCA (9 kb)-SV40TAG and PSCA (6 kb)-SV40TAG) (Brinster *et al* 1984 Cell 37:367-379).

- The chimeric vectors were used to generate a line of founder transgenic mice. Linearized chimeric vectors were microinjected into fertilized mouse eggs derived from intercrosses of C57BL/6X C3H hybrid mice. Founder mice that carried the chimeric vector were identified by Southern analysis of tail DNA, using GFP cDNA or SV40 genomic DNA as a probe. The number of founders of each transgenic mouse line is indicated on the right panel of Figure 45.

Example 15

- 25 **hPSCA upstream sequences drives expression of transgene in transgenic mice**

- Two independent founder mice carrying PSCA (9 kb)-GFP transgene were bred to Balb/c mice to obtain their offspring. At age of 8 weeks and 12 weeks, male and female transgenic or non-transgenic littermates were sacrificed. After sacrifice, all urogenital and other tissues were tested for GFP expression by observing the fixed tissues under fluorescent illumination. The results

shown in Figure 46 show green fluorescent images of prostate, bladder and skin tissues from a non transgenic and a transgenic mouse. One out of two founder lines expressed GFP protein in prostate, bladder and skin (Figure 46). Tissues that did not express GFP include: seminal vesicle, liver, stomach, kidney, lung, brain, testis, pancreas, heart, skeletal muscle, small intestine, colon, placenta.

Example 16

Transcript expression pattern of PSCA in human and mouse tissue

The upper panel of Figure 47 shows a human multiple tissue Northern blot (obtained from Clontech), probed with a full length human PSCA cDNA probe. The results demonstrate that human PSCA transcripts are abundant in prostate, and less abundant but readily detectable in placenta, but not detectable in spleen, thymus, testis ovary, small intestine, colon, peripheral blood leukocytes (PBL), heart, brain, lung, liver, muscle, kidney and pancreas.

The lower panel of Figure 47 shows an ethidium bromide-stained agarose gel of RT-PCR analysis of murine PSCA transcript expression patterns in various mouse tissues. The RT-PCR was prepared using Ultraspec.RNA (Biotex), and cDNA cycle kit (Invitrogen). Primers corresponding to a region within exon 1 and exon 3 of PSCA were used to amplify a 320 bp fragment. The exon 1 primer sequence is as follows:

5' primer: 5'-TTCTCCTGCTGGCCACCTAC-3'. The exon 3 primer sequence is as follows:

3' primer: 5'-GCAGCTCATCCCTTCACAAT-3'. As a control, to demonstrate the integrity of the RNA samples isolated from the various mouse tissues, a 300 bp G3PD fragment was amplified.

The results shown in the lower panel of Figure 47 demonstrate that murine PSCA transcripts are detectable in dorsal/lateral prostate, ventral prostate, bladder, stomach (cardiac, body and pyloric), and skin. In contrast, murine PSCA transcripts are not detectable in anterior prostate, ventral prostate, seminal vesicle, urethra, testis, kidney, duodenum, small intestine, colon, salivary gland,

spleen, thymus, bone marrow, skeletal muscle, heart, brain, eye, lung and liver. The G3PDH results demonstrate that the transcripts isolated from various mouse tissue were intact.

- 5 The present invention is not to be limited in scope by the embodiments disclosed herein, which are intended as single illustrations of individual aspects of the invention, and any which are functionally equivalent are within the scope of the invention. Various modifications to the models and methods of the invention, in addition to those described herein, will become apparent to those skilled in the art from the foregoing description and teachings, and are similarly intended to fall
- 10 within the scope of the invention. Such modifications or other embodiments can be practiced without departing from the true scope and spirit of the invention.

What is claimed:

1. A nucleic acid molecule comprising a PSCA upstream regulatory region.
- 5 2. The nucleic acid molecule of claim 1, wherein the PSCA upstream regulatory region is derived from a human.
3. The nucleic acid molecule of claim 1, wherein the PSCA upstream regulatory region is derived from a mouse.
- 10 4. The nucleic acid molecule of claim 1, wherein the PSCA upstream regulatory region comprises a sequence having promoter activity.
5. The nucleic acid molecule of claim 1, wherein the PSCA upstream regulatory region comprises a sequence having enhancer activity.
- 15 6. The nucleic acid molecule of claim 1 designated ATCC No. PTA-80.
7. A BglI-HindIII fragment of ATCC No. PTA-80 as shown in figure 42.
- 20 8. A BamHI-HindIII fragment of ATCC No. PTA-80 as shown in figure 42.
9. A KpnI-HindIII fragment of ATCC No. PTA-80 as shown in figure 42..
- 25 10. The nucleic acid molecule of claim 1 further comprising a transgene operatively linked to the PSCA upstream regulatory region.
11. The nucleic acid molecule of claim 10, wherein the transgene encodes a reporter gene.

12. The nucleic acid molecule of claim 10, wherein the transgene encodes a gene product that induces tumor formation.
13. The nucleic acid molecule of claim 10, wherein the transgene is a therapeutic gene selected from a group consisting of a toxin gene, a cytokine gene, an interferon gene, a growth factor gene, a tumor suppressor gene, a prodrug enzyme gene, antisense RNA, an antibody gene, or an oncostatin gene.
14. A vector comprising the nucleic acid molecule of claim 1.
15. The vector of claim 14, further comprising a sequence encoding a PSCA protein operatively linked to the PSCA upstream regulatory region.
16. The vector of claim 14, further comprising a transgene operatively linked to the PSCA upstream regulatory region.
17. A host-vector system comprising the vector of claim 14 transfected into a compatible host cell.
18. The host-vector system of claim 17, wherein the host cell is a prostate cell.
19. A method for producing a gene product, the method comprising:
 - a) growing the host-vector system of claim 18 so as to produce the gene product; and
 - b) recovering the gene product so produced.
20. A protein produced by the method of claim 19.
21. A transgenic animal having germ and somatic cells having an introduced nucleic acid molecule comprising an oncogene operatively linked to a PSCA upstream regulatory region, the introduced nucleic acid molecule being effective for expression of the oncogene in the

tissues of the mouse and being effective for inducing tumor formation in the urogenital tissues of the mouse, wherein the nucleic acid was introduced into the mouse, or an ancestor of the mouse, at an embryonic stage.

- 5 22. A method for killing prostate tumor cells, the method comprising introducing the vector of claim 16 to a prostate tumor cell, so that the vector expresses a functional molecule in a sufficient amount to kill the tumor cells.
23. A method for killing prostate tumor cells, the method comprising:
- 10 a) introducing the vector of claim 16 to a prostate tumor cell, wherein the transgene encodes a prodrug enzyme;
- b) administering a prodrug that will be converted into a toxic substance by the prodrug enzyme expressed by the vector, thereby killing the tumor cell.

PSCA: PROSTATE STEM CELL ANTIGEN AND USES THEREOF

ABSTRACT OF THE DISCLOSURE

- 5 The invention provides a novel prostate cell-surface antigen, designated Prostate Stem Cell Antigen (PSCA), which is widely over-expressed across all stages of prostate cancer, including high grade prostatic intraepithelial neoplasia (PIN), androgen-dependent and androgen-independent prostate tumors.

1 agggagagggc agtgaccatg aaggctgtgc tgcctgccct gtagatggca
 51 ggcttggccc tgcagccagg cactgccctg ctgtgctact cctgcaaagc
 101 ccaggtgagc aacgaggact gccctgcaggt ggagaactgc acccagctgg
 151 gggagcagtg ctggaccgcg cgcaiccgcg cagtggcct cctgaccgtc
 201 atcagcaaag gctgcagctt gaactgcgtg gatgactcac aggactacta
 251 cgtgggcaag aagaacatca cgtgctgtga caccgacttg tgcaacgcca
 301 gcgggggcca tgcctgcag cgggctgccg ccacccctgc gctgctccct
 351 gcactcggcc tgcctgctcg gggaccggc cagctatagg ctctgggggg
 401 ccccgctgca gccacactg ggtgtgtgc cccaggcctt tgtgccactc
 451 ctacagaac ctggcccagt gggagcctgt cctggctcct gaggcacatc
 501 ctacgcaag ttgaccatg tatgttgca cccctttcc ccaaccctg
 551 acctcccat gggcctttc caggattccn accnggcaga tcagttagg
 601 tganacanat ccgctgcag atggccctc caaccnttn tgttgnrtg
 651 tccatggccc agcatttcc accttaacc ctgtgttcag gcacttttc
 701 ccccaggaag cctccctgc ccacccan tatgaattga gccaggttg
 751 gtccgtggcg tccccgcac ccagcagggg acaggcaatc aggagggccc
 801 agtaaaaggc gagatgaagt ggactgagta gaactggagg acagaggtg
 851 acgtgagtc ctgggagtc ccagagagtg ggcctggagg cctggagga
 901 ggggcccagg ctacatag tggggtccc gaatggcagc ctgagcaccg
 951 cgtaggccct taataaacac ctgtggatg agccaaaaaa aaaaaaaa

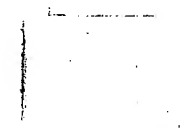


FIGURE 1A



MKAVLLALLMAGLALQPGTALLCYSCAQVSNEDECLQV
ENCTQLGEQCWTARIRAVGLLTVISKGCSLNCVDDS
QDYVVGKKNITCCDTLDCNASGAHALQPAAAILALLPAL
GLLLWGPGQL

FIGURE 1B

ATGAAGACAGATTTTTTTTATCCTGCTGGCCACCTACTTAGCCCTGCATCCAGGTGCTGCT
1 60
TACTTCTGTCAAAAAAATAGGACGACCGGTGGATGAATCGGGACGTAGGTCCACGACGA
M K T V F F I L L A T Y L A L H P G A A -
CTGCAGTGCTATTTCATGCACAGCACAGATGAACAACAGAGACTGTCTGAATGTACAGAAC
61 120
GACGTCACGATAAGTACGTGTCTGTCTACTTGTGTCTCTGACAGACTTACATGTCTTG
L Q C Y S C T A Q M N N R D C L N V Q N -
TGCAGCCTGGACCAGCACAGTTGCTTTACATCGCGCATCCGGGCCATTGGACTCGTGACA
121 180
ACGTCGGACCTGGTCTGTCAACGAAATGTAGCGCGTAGGCCCGGTAACCTGAGCACTGT
C S L D Q H S C F T S R I R A I G L V T -
GTTATCAGTAAGGGCTGCAGCTCACAGTGTGAGGATGACTCGGAGAACTACTATTTGGGC
181 240
CAATAGTCATTCCCGACGTCGAGTGTCACTCCTACTGAGCCTCTTGATGATAAACCCG
V I S K G C S S Q C E D D S E N Y Y L G -
AAGAAGAACATCACGTGCTGCTACTCTGACCTGTGCAATGTCAACGGGGCCCAACCCCTG
241 300
TTCTTCTTGTAGTGACGACGATGAGACTGGACACGTTACAGTTGCCCGGGTGTGGGAC
K K N I T C C Y S D L C N V N G A H T L -
AAGCCACCCACCACCCTGGGGCTGCTGACCGTGCTCTGCAGCCTGTTGCTGTGGGGCTCC
301 360
TTCGGTGGGTGGTGGGACCCCGACGACTGGCACGAGACGTCGGACAACGACACCCCGAGG
K P P T T L G L L T V L C S L L L W G S -
AGCCGTCTGTAGGCTCTGGGAGAGCCTACCATAGCCCGATTGTGAAGGGATGAGCTGCAC
361 420
TCGGCAGACATCCGAGACCCTCTCGGATGGTATCGGGCTAACACTTCCCTACTCGACGTG
S R L *
TCCACCCCAACCCCAACACAGG
421 441
AGGTGGGGTGGGGTGTGTCC

FIGURE 2



1 M K I F L P V T L R A M L N G V S R A S S hSCA-2
1 M K A V L L A L L M A G E A L O P T G T A hPSCA
1 M K T V L F L L L A T Y T A L R P G A A mPSCA

21 L M C F S C L N Q K S N L Y C E K P T I
21 L L C Y S C K A Q V S N S D C L Q V E N
21 L Q C Y S C T A Q M N N R D C L N V Q N

41 C S D Q D N Y C V T V S A S A G I G N L
41 C T O L G E Q C W T A R I R A V G L L T
41 C S L D Q H S C F T S R I R A I G L V T

61 V T F G H S L S K T C S P A C P I P E G
61 V - - - - I S K G C S L N C V D D S Q
61 V - - - - I S K G C S S Q C E D D S E

81 V N V G V A S M G I S C C Q S F L C N F
76 D Y Y V G K K - N L L C C O T D L C N A
76 N Y Y L G K K - N L T C C Y S D L C N V

101 S I A A D G G L R A S V T L E G A G E L L
95 S G A B A L O P A A A L L A L L P A F G
95 N G A B T L X P P T T L G L L T V L C S

121 S L L P A L L R F G P
115 L L L W G P G O L - -
115 L L L W G S S R L - -

FIGURE 3

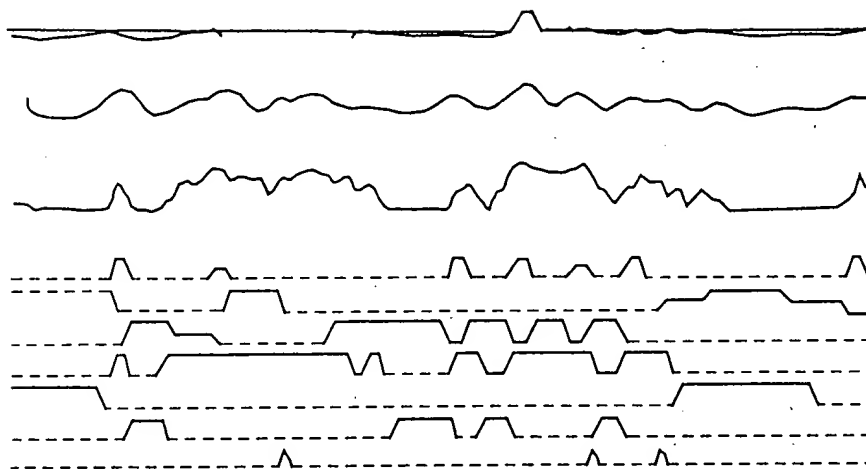


FIGURE 4

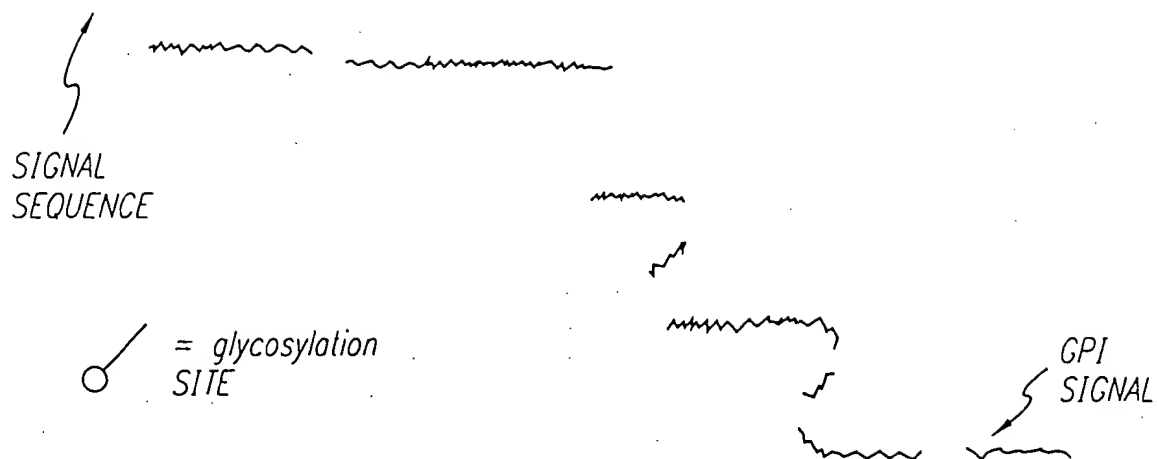
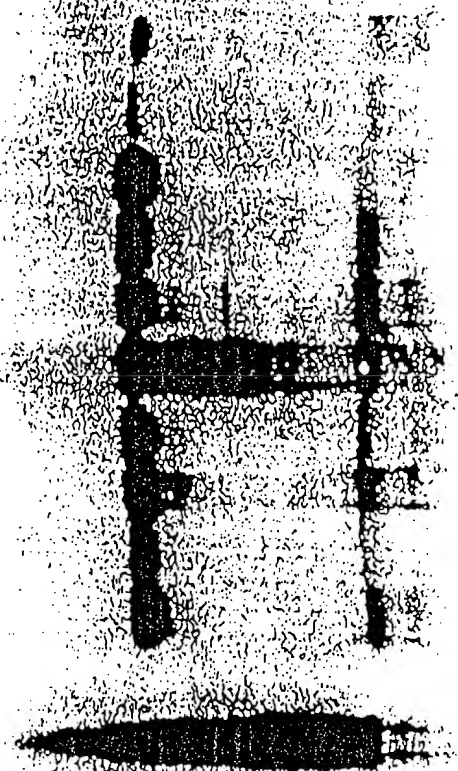


FIGURE 5



Western BSCA
Suppose to be 80% AB
Normal tissue
1hr exp

1G8
1:100



prostate (Kamer)
prostate (Baker)
prostate (Glick)
Bladder (Kamer)
Bladder (Glick)
Bladder (Kob)
Kidney (MAG)
Kidney (WUZ)
Testis
Sm. Intest.

LA PC9

FIGURE 6

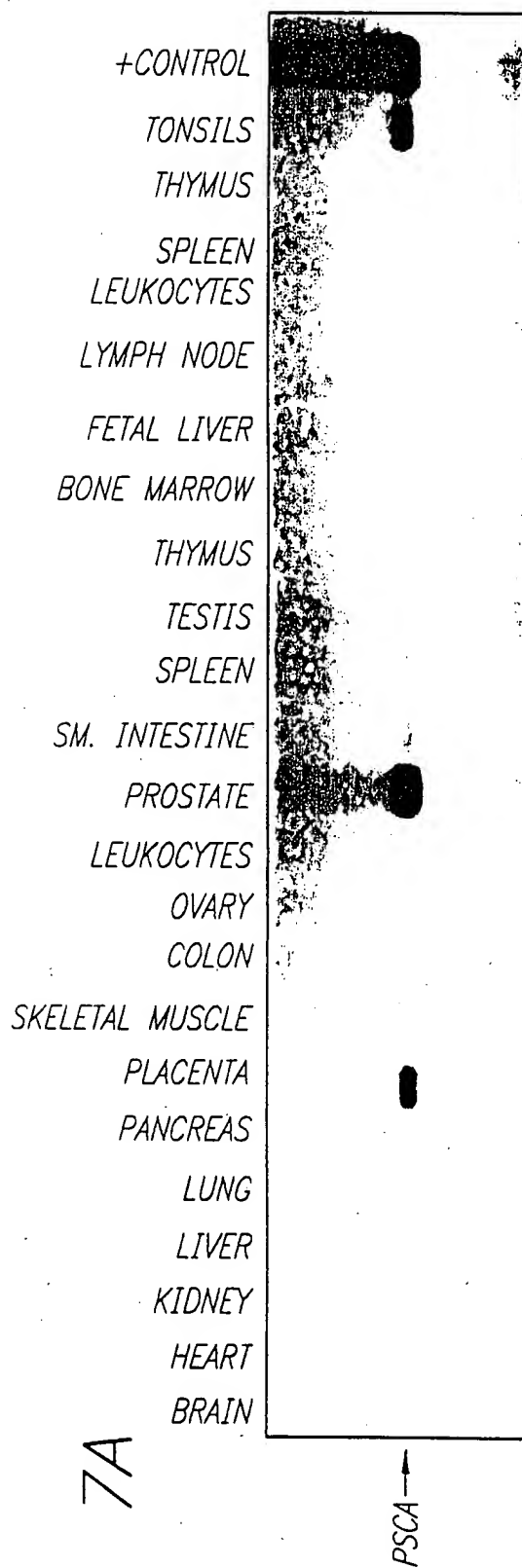


FIG. 7A

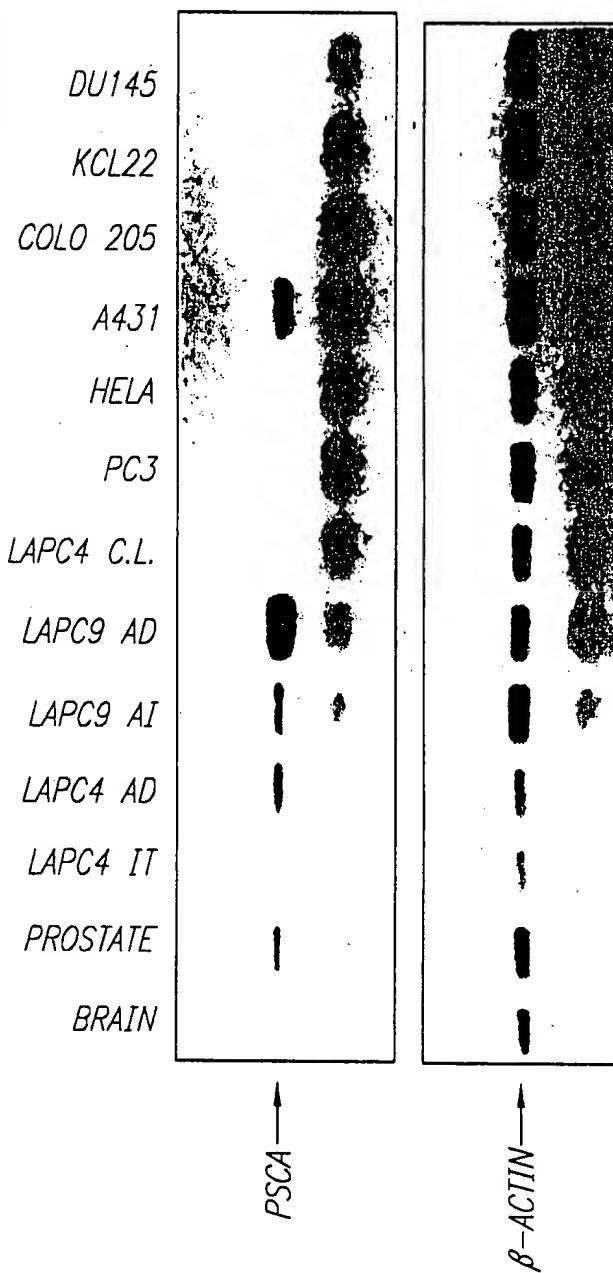


FIG. 7B

FIG. 8A

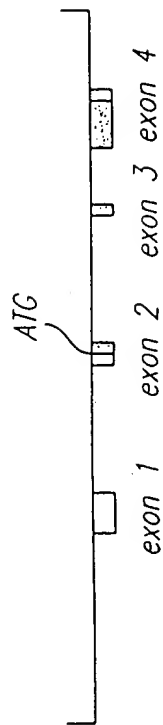


FIG. 8B

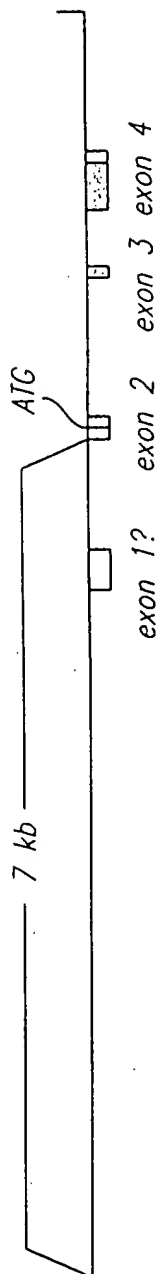


FIG. 8C

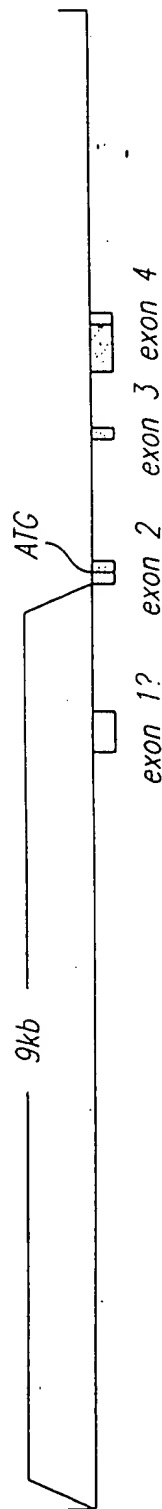


FIGURE 8





PSCA / PSA Expression in Benign Prostate vs. Prostate Cancer Xenograft

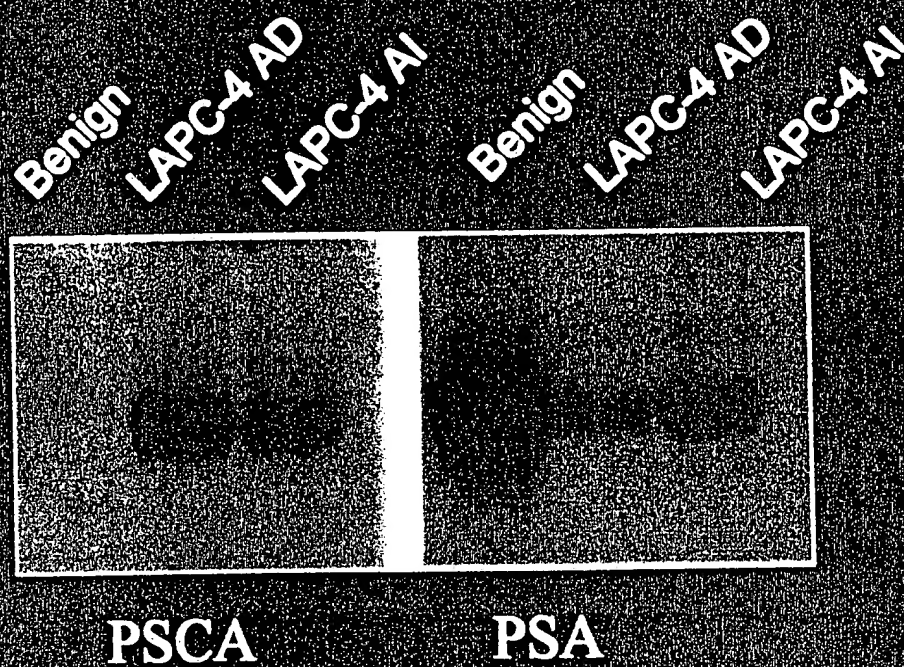


FIGURE 9A

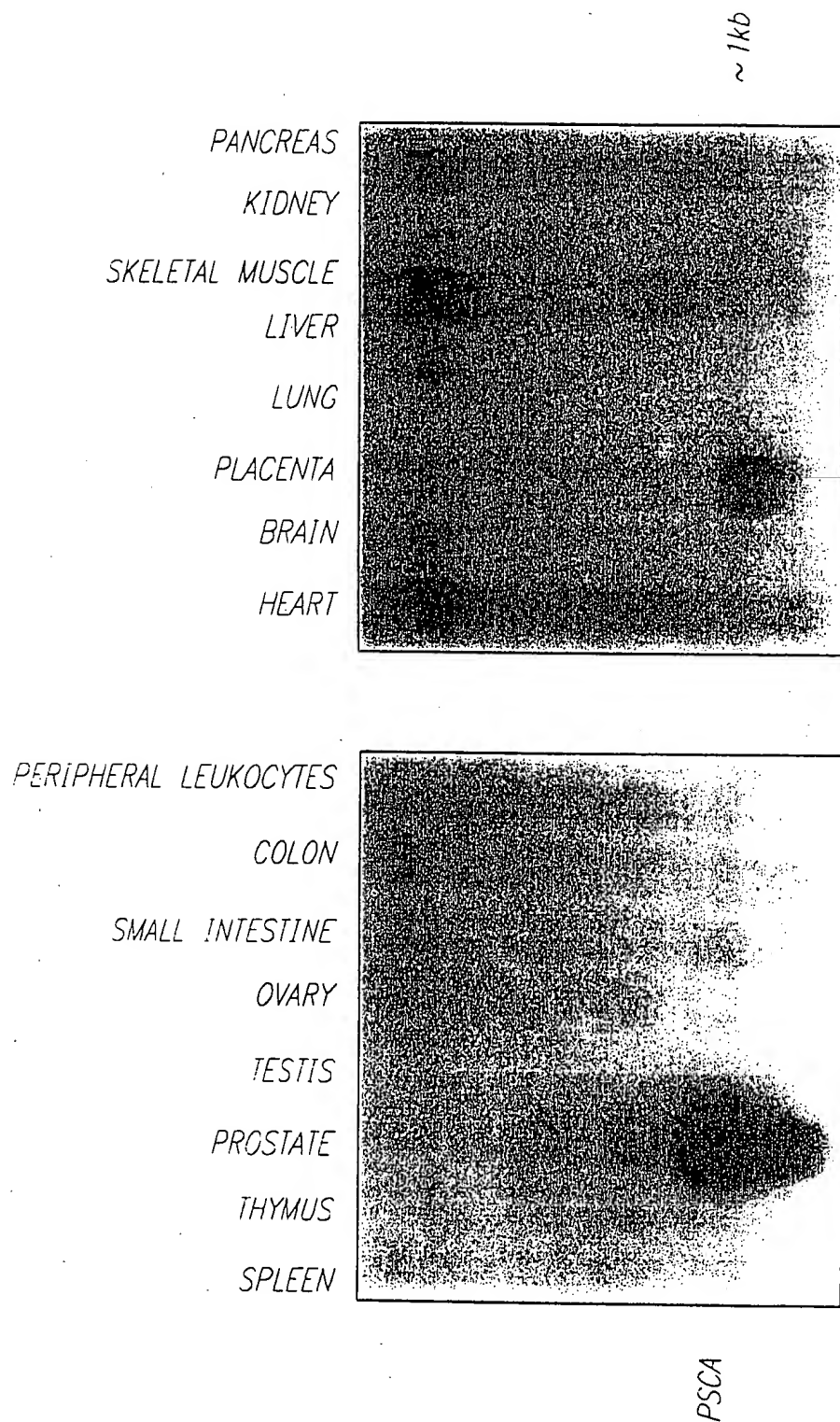
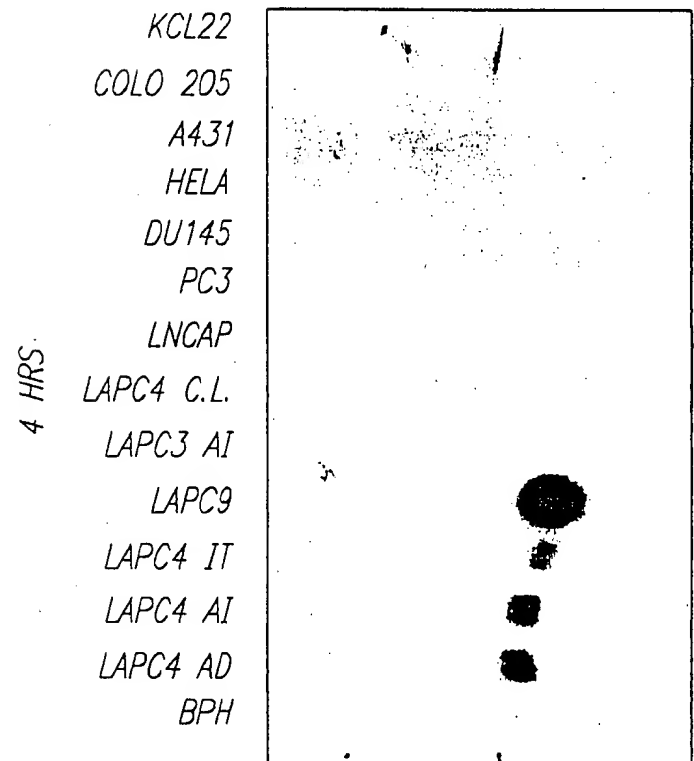
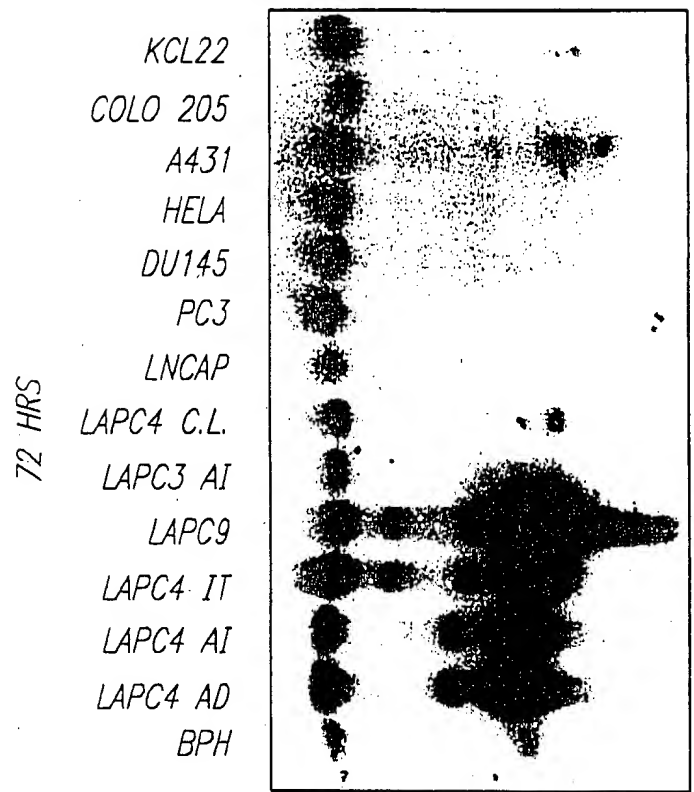


FIG. 9B



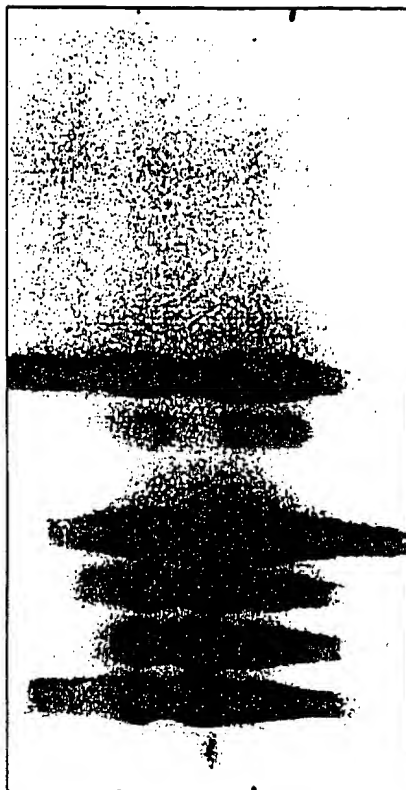
PSCA



FIG. 10-1

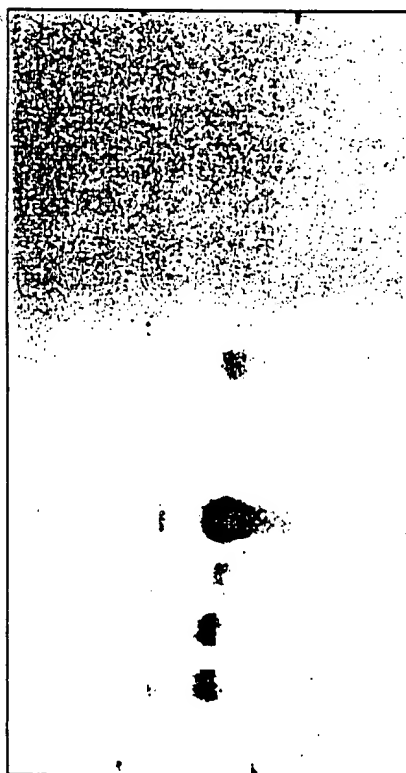
72 HRS

KCL22
 COLO 205
 A431
 HELA
 DU145
 PC3
 LNCAP
 LAPC4 C.L.
 LAPC3 AI
 LAPC9
 LAPC4 IT
 LAPC4 AI
 LAPC4 AD
 BPH



4 HRS

KCL22
 COLO 205
 A431
 HELA
 DU145
 PC3
 LNCAP
 LAPC4 C.L.
 LAPC3 AI
 LAPC9
 LAPC4 IT
 LAPC4 AI
 LAPC4 AD
 BPH



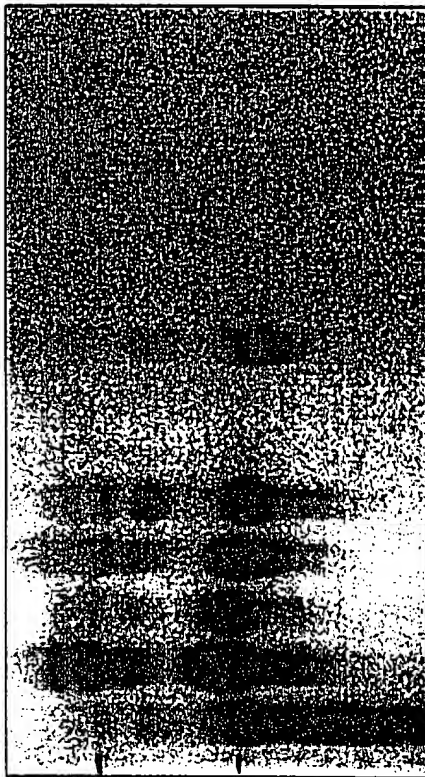
PSM

FIG. 10-2



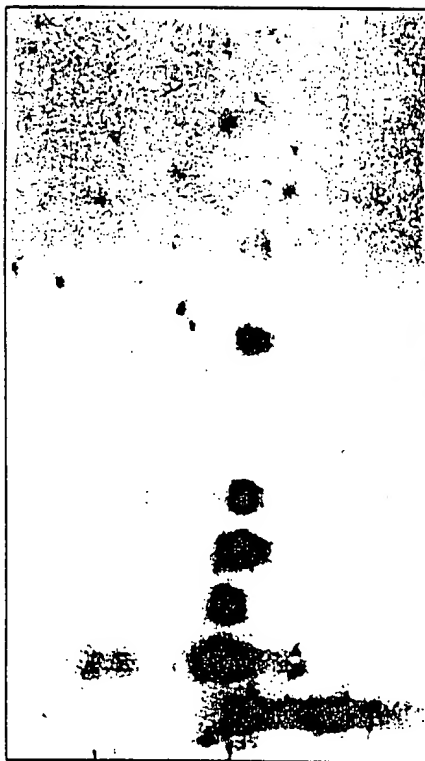
72 HRS

KCL22
COLO 205
A431
HELA
DU145
PC3
LNCAP
LAPC4 C.L.
LAPC3 AI
LAPC9
LAPC4 IT
LAPC4 AI
LAPC4 AD
BPH

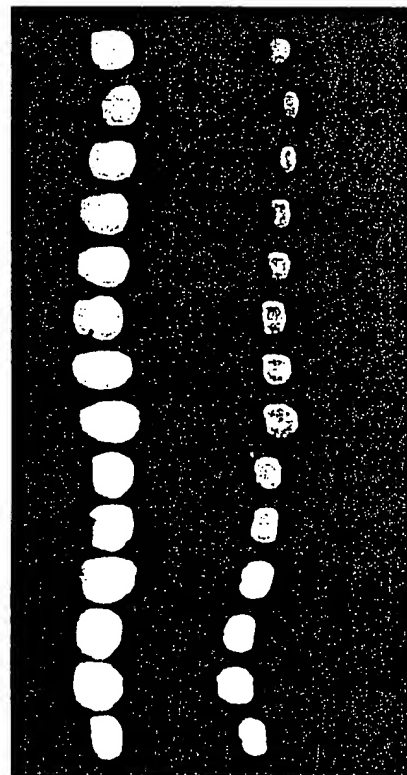


4 HRS

KCL22
COLO 205
A431
HELA
DU145
PC3
LNCAP
LAPC4 C.L.
LAPC3 AI
LAPC9
LAPC4 IT
LAPC4 AI
LAPC4 AD
BPH



PSA



ETBR

FIG. 10-3

FIG. 11A

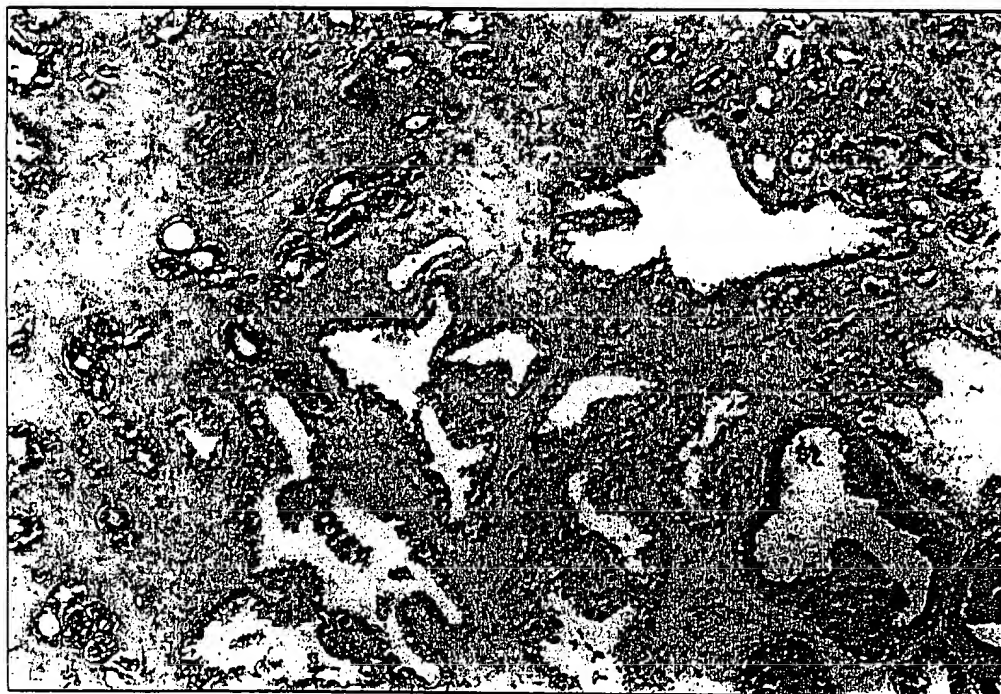


FIG. 11B

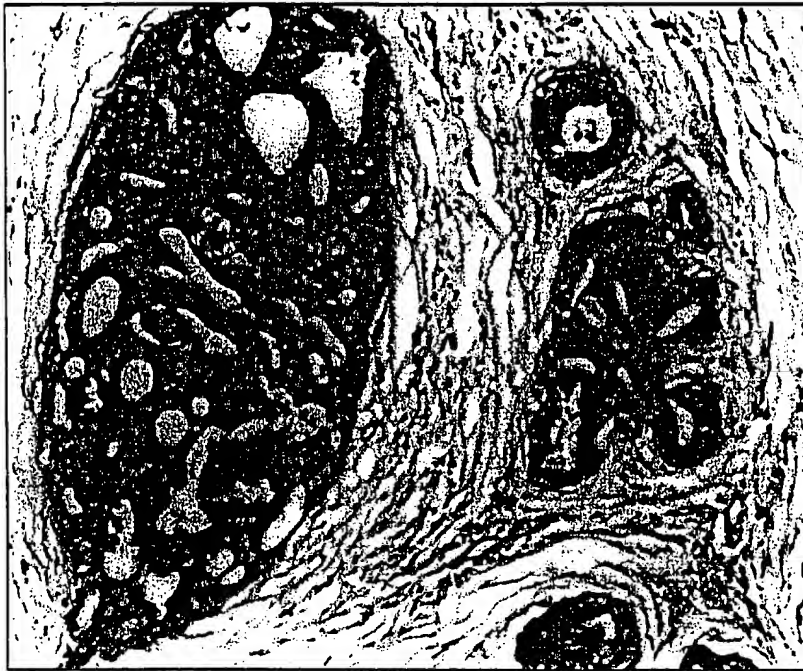


FIG. 11C

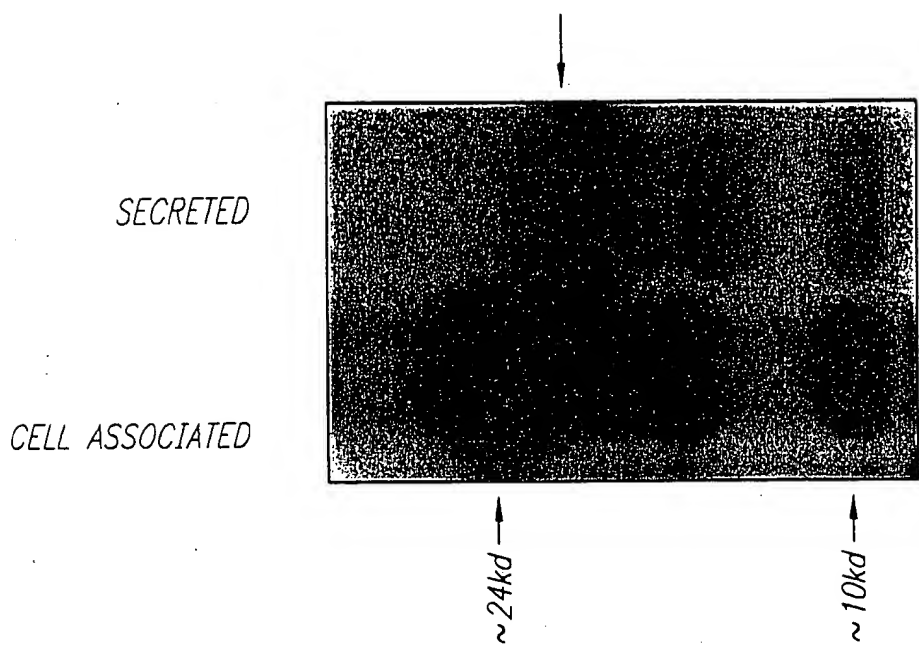


FIG. 12B

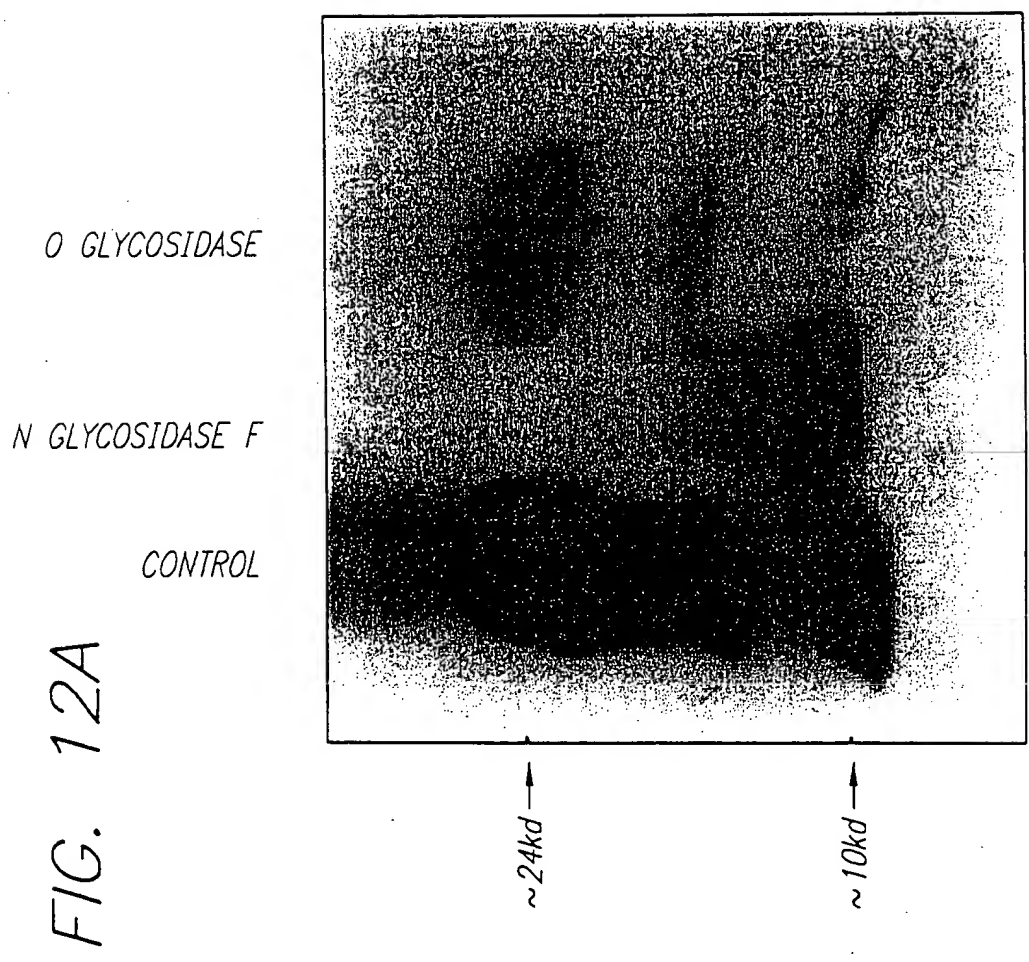
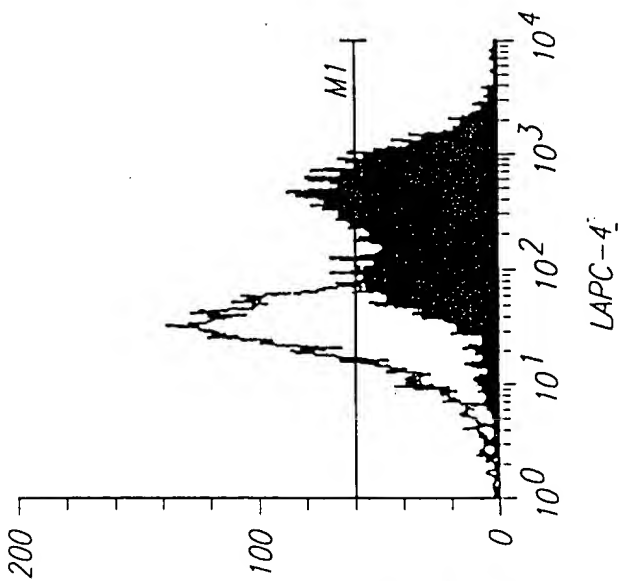
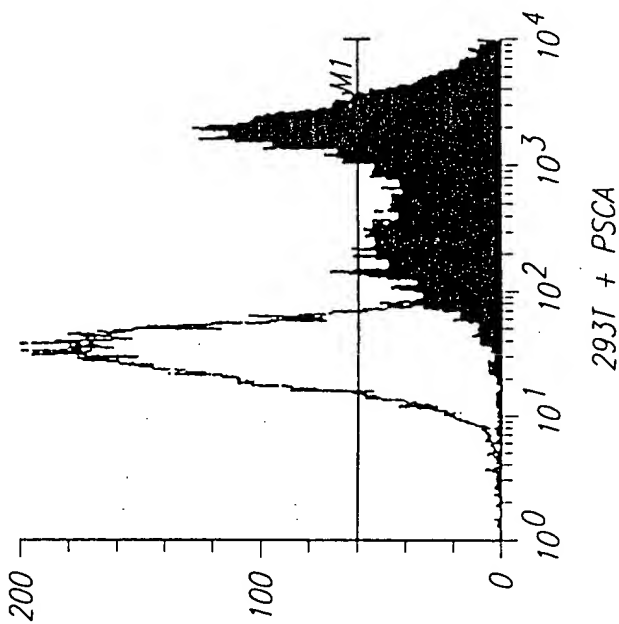


FIG. 12A

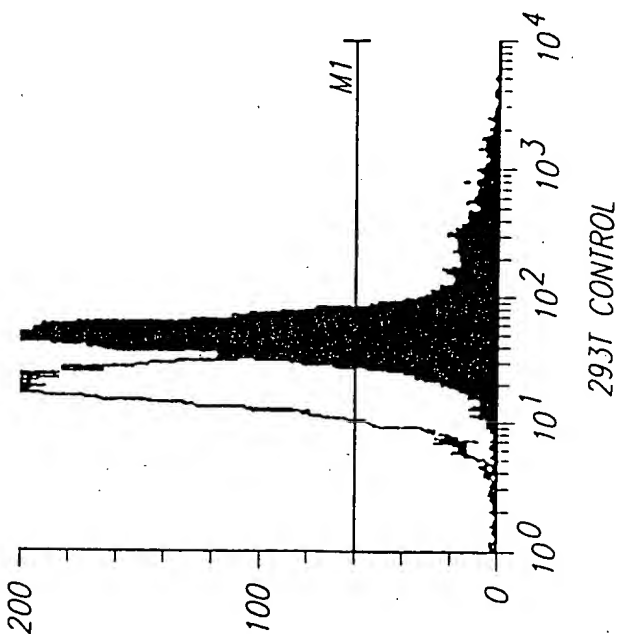
293T971003012\FL2-H\FL2-Height



293T971003010\FL2-H\FL2-Height



293T9710030008\FL2-H\FL2-Height



293T CONTROL

293T + PSCA

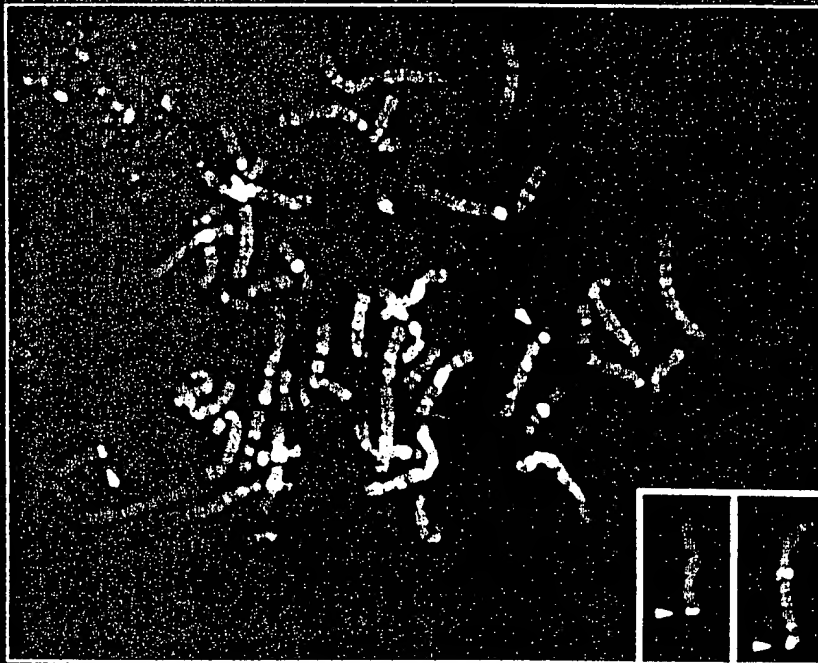
LAPC-4

FIGURE 12C





PSCA Maps to Chromosome 8q24.2



Fluorescent
in Situ Hybridization
Analysis of PSCA

FIGURE 13

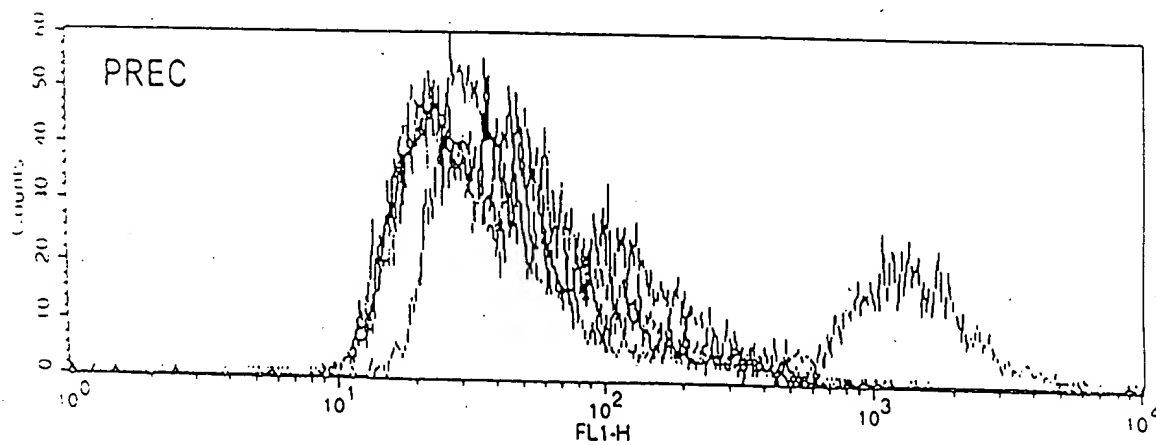
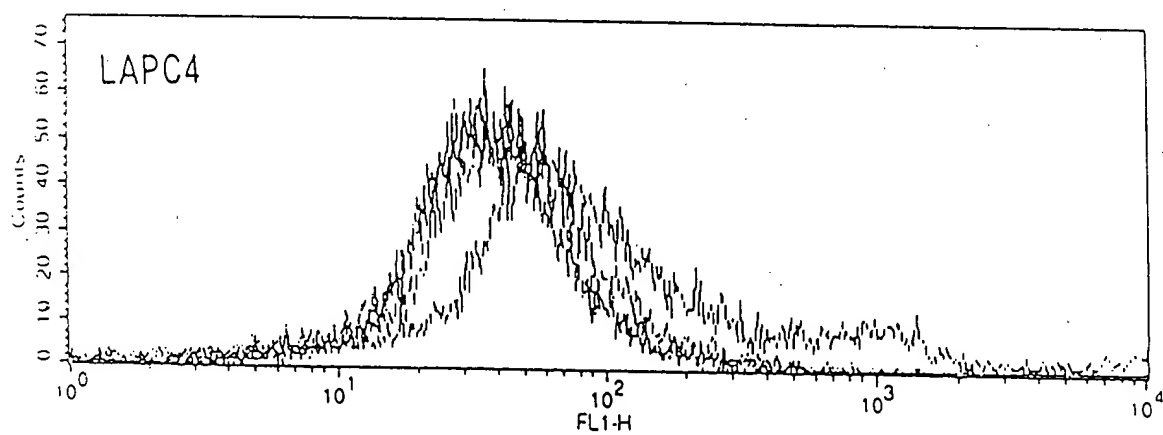
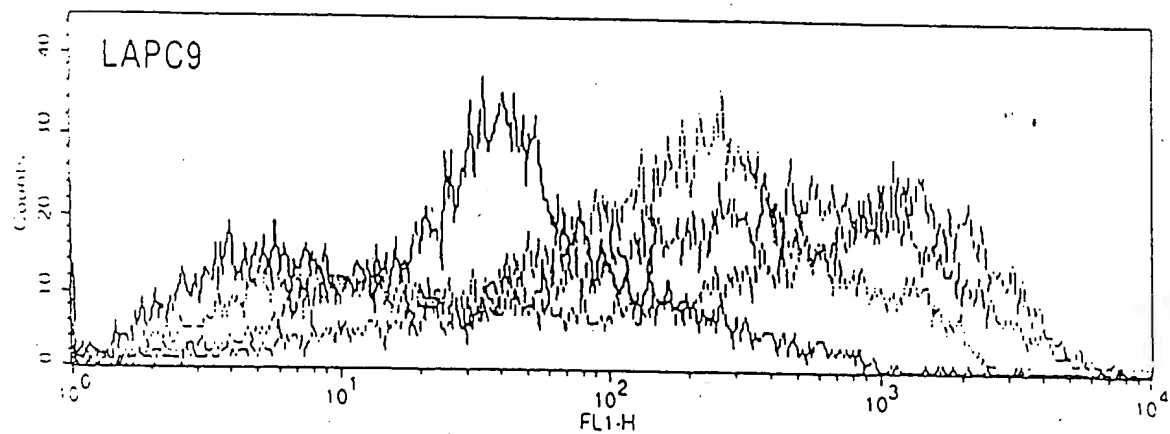


FIGURE 14

A

Epitope map

mAb	Isotype	EL (18-98)	N (2-50)	M (46-109)	C (85-123)
1G8	IgG1 k	2.039	0.007	0.628	0.000
2H9	IgG1 k	1.318	0.863	0.032	0.021
3C5	IgG2a k	2.893	1.965	0.016	0.005
3E6	IgG3 k	0.328	0.024	0.069	0.370
4A10	IgG2a k	2.039	1.315	0.000	0.014
2A2	IgG2a k	1.366	0.733	0.010	0.003
3G3	IgG2a k	2.805	1.731	0.004	0.000

B

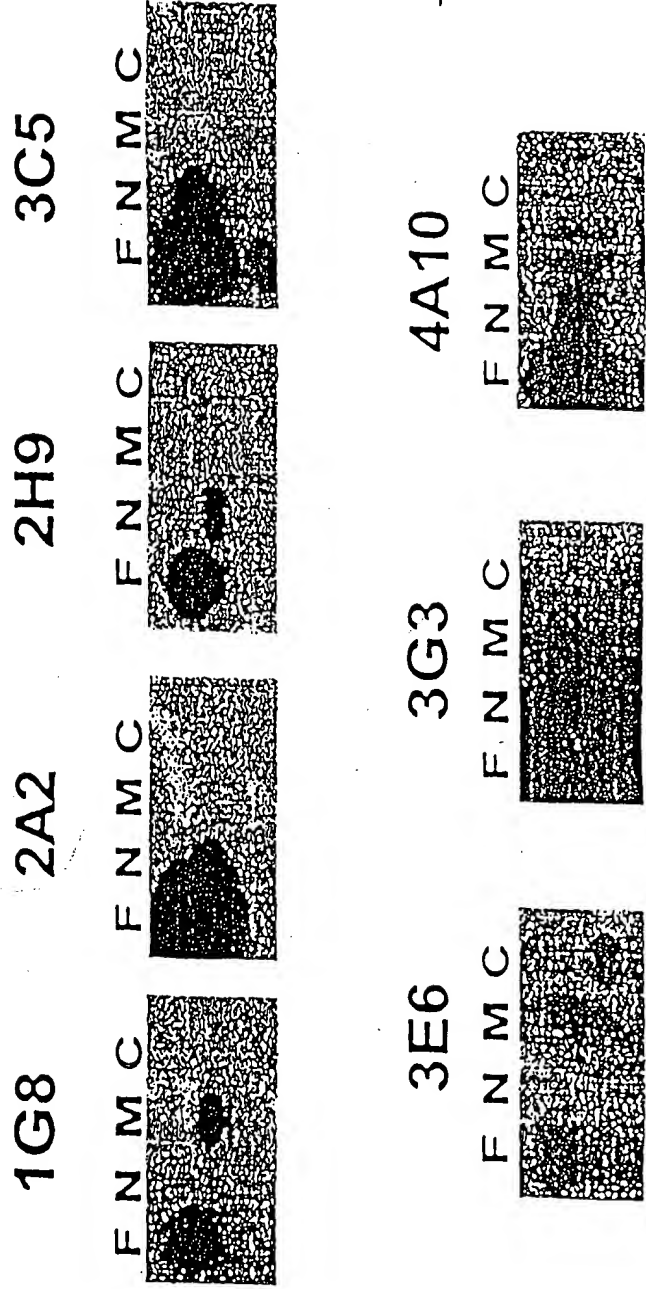


FIGURE 15





Prostate Stem Cell Antigen (PSCA) is a GPI-anchored Protein

1	MSK	I	F	H	P	V	D	S	L	A	L	L	I	G	T	E	R	A	S	S		hSCA-2
1	MSK	A	V	H	L	A	A	T	M	A	G	L	A	L	O	P	G	T	A		hPSCA	
1	MSK	T	D	H	F	L	D	S	A	T	T	L	A	L	H	P	G	A	A		mPSCA	
21	M	C	F	S	C	L	N	Q	K	S	N	L	Y	C	L	K	P	T	I			
21	L	C	S	C	K	A	Q	V	S	N	E	D	C	L	Q	V	E	N	*			
21	Q	C	S	C	T	A	O	M	N	N	R	D	C	L	N	V	C	N	*			
41	C	S	D	D	N	Y	C	V	T	V	S	A	S	A	G	I	U	N	L			
41	C	T	L	D	N	C	W	T	A	R	I	R	A	V	G	L	I	T				
41	C	S	L	D	D	S	C	F	T	R	I	R	A	V	G	L	I	T				
61	V	T	F	H	B	L	S	K	T	C	S	P	A	C	P	I	F	E	D			
61	V	-	-	-	-	I	S	K	G	C	S	L	N	C	V	D	D	S				
61	V	-	-	-	-	I	S	K	G	C	S	L	N	C	V	D	D	S				
81	V	N	V	D	V	A	S	M	G	T	S	C	C	Q	S	F	L	C	N	*		
76	D	V	V	V	G	K	K	-	N	I	T	C	C	D	T	D	L	C	N	*		
76	N	V	V	V	G	K	K	-	N	I	T	C	C	V	S	D	L	C	N	*		
101	S	A	T	D	G	L	R	A	N	T	L	L	L	A	L	L	L	L				
95	S	A	T	D	G	L	R	A	N	T	L	L	L	A	L	L	L	L				
95	N	G	A	N	T	L	K	P	P	T	L	L	L	T	V	L	C					
121	S	A	T	D	G	L	R	A	N	T	L	L	L	A	L	L	L	L				
115	S	A	T	D	G	L	R	A	N	T	L	L	L	A	L	L	L	L				
115	S	A	T	D	G	L	R	A	N	T	L	L	L	A	L	L	L	L				

(Reiter, R.E., et al., 1997. *PNAS*)

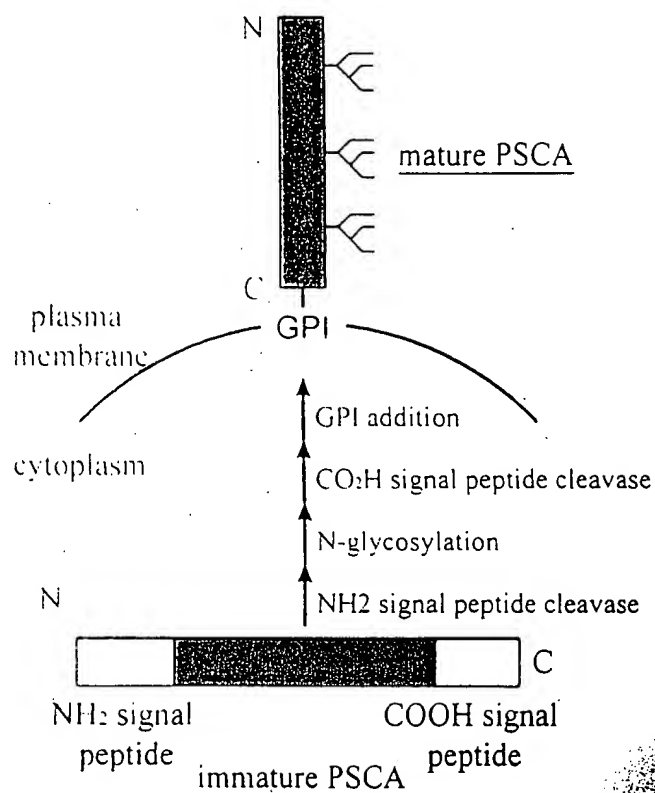


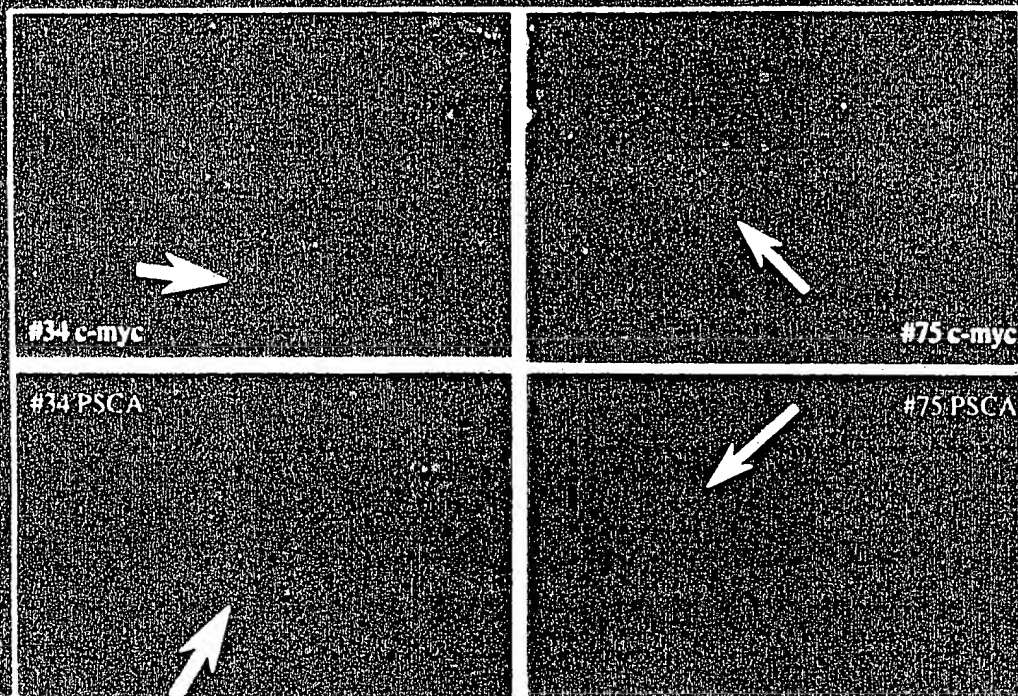
FIGURE 16



FISH Analysis of PSCA and c-myc in Prostate Cancer

Gain Chromosome 8

Amplification



R. Jenkins

FIGURE 17

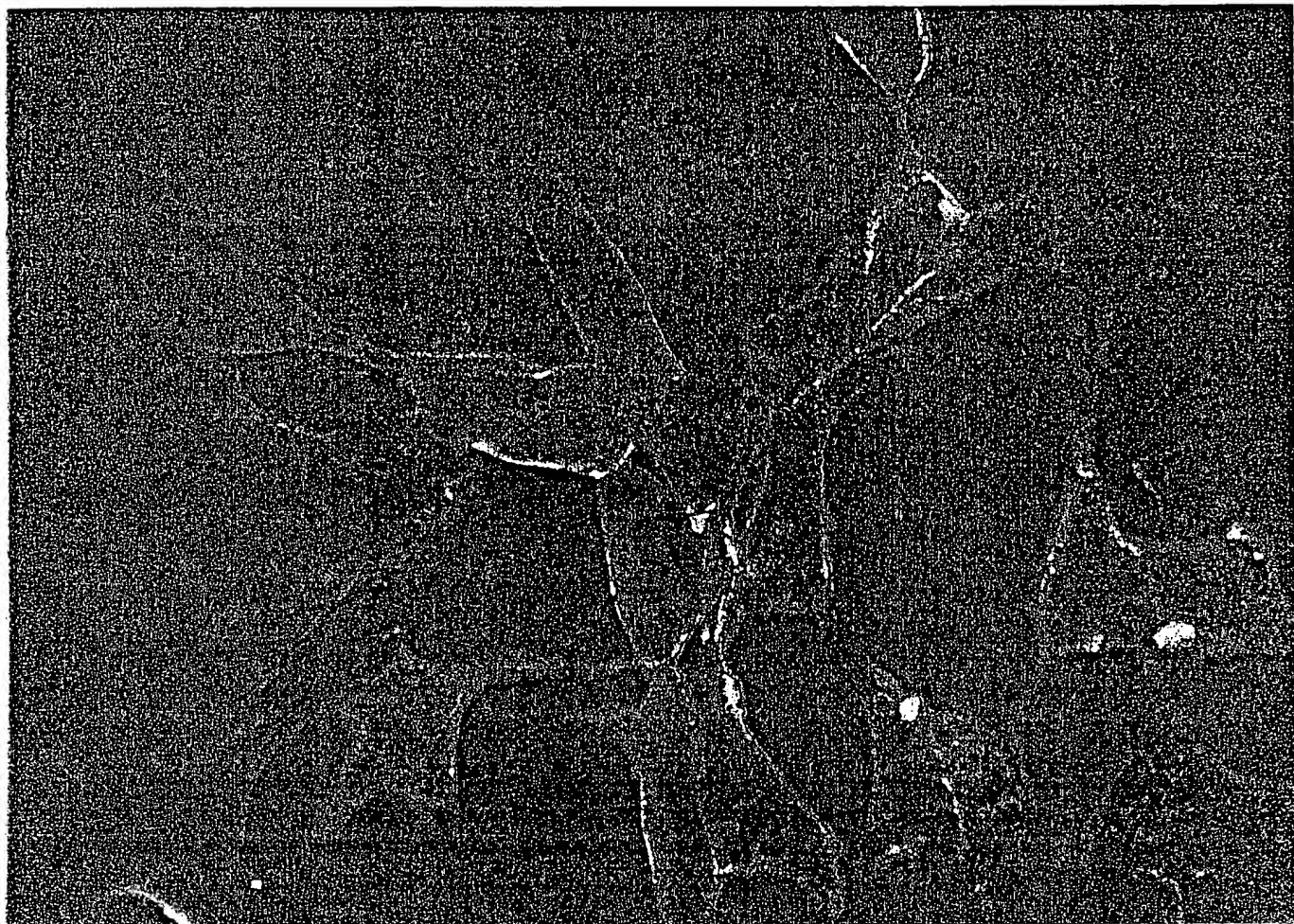


FIGURE 18

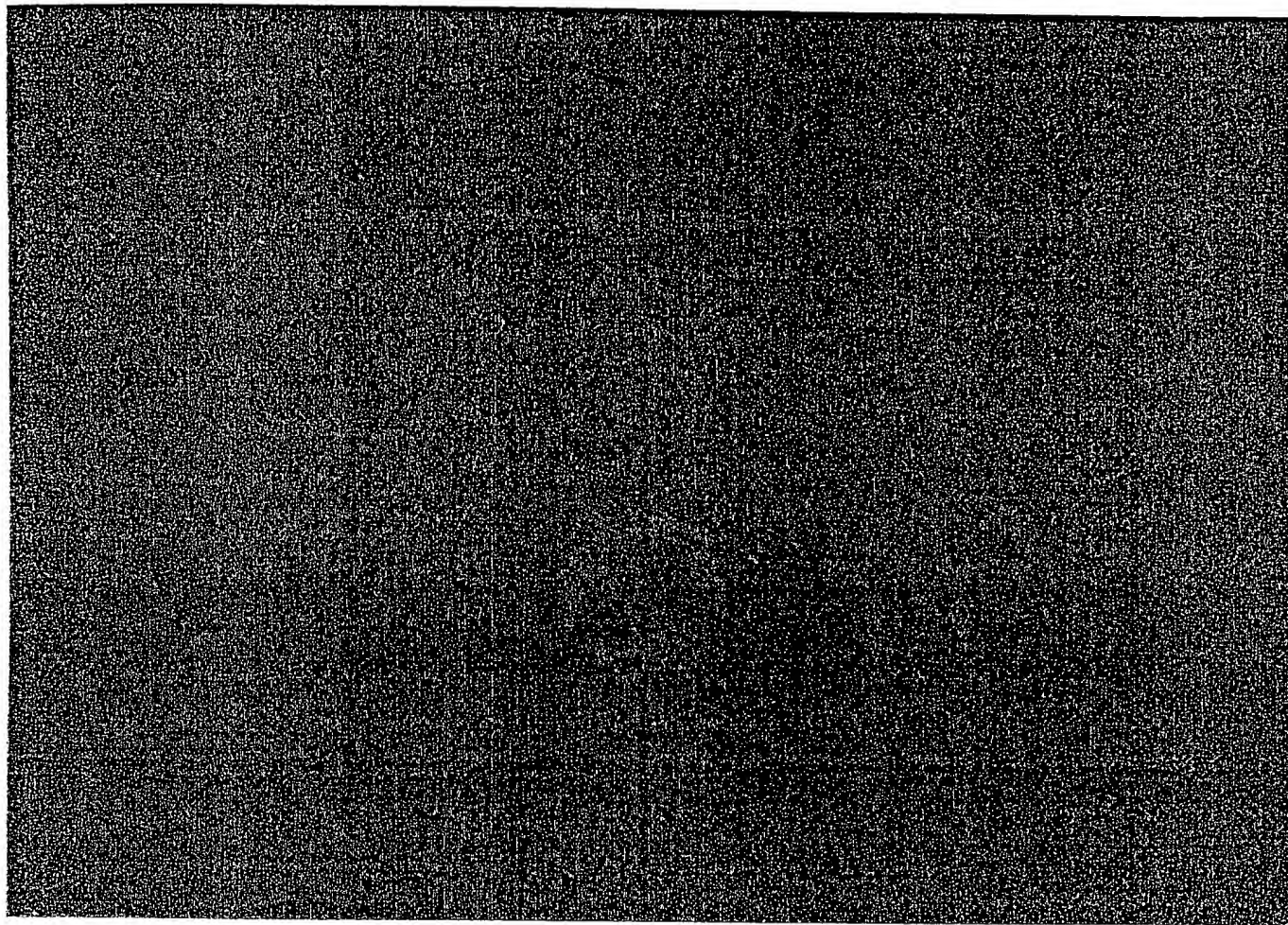


FIGURE 19

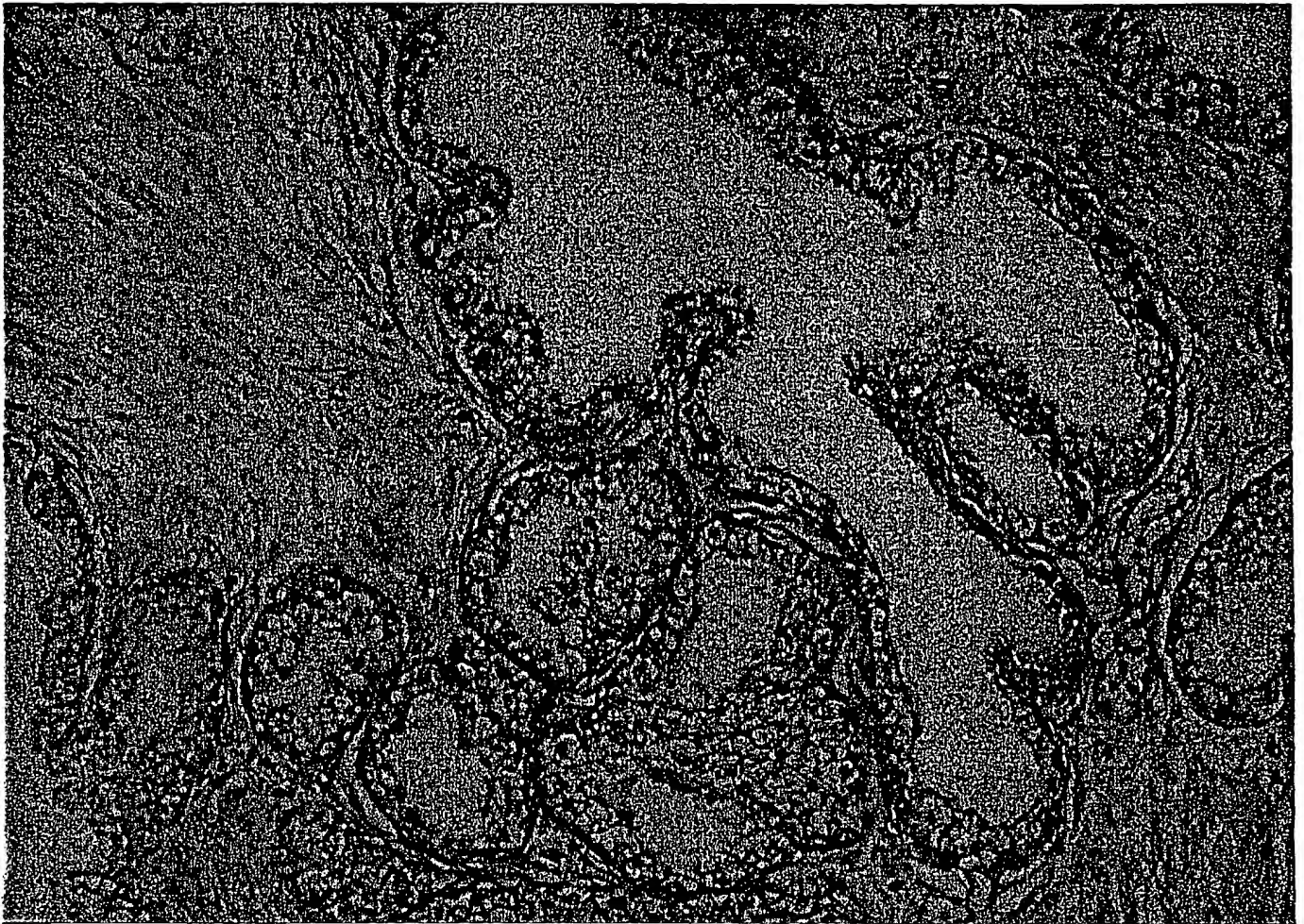


FIGURE 20



PSCA Immunostaining of Primary Tumors

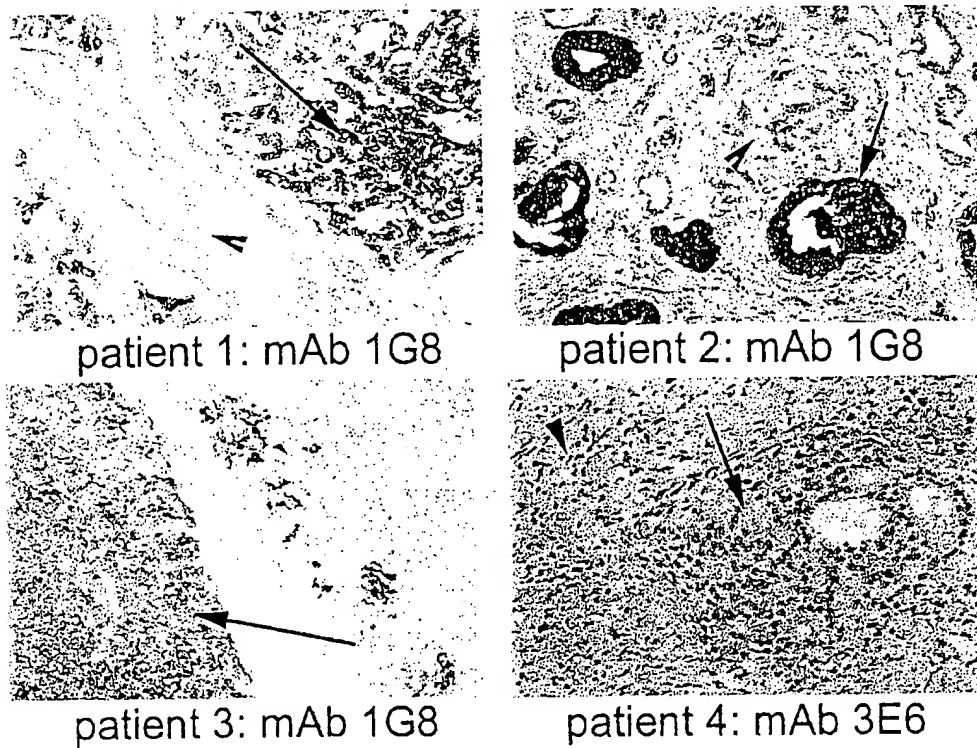


FIGURE 21



FIGURE 22

U.S. DEPARTMENT OF
HEALTH, EDUCATION &
WELFARE
OFFICE OF
RESEARCH & DEVELOPMENT
NATIONAL INSTITUTE OF
ENVIRONMENTAL HEALTH
SCIENCES
BETHESDA, MARYLAND
20892

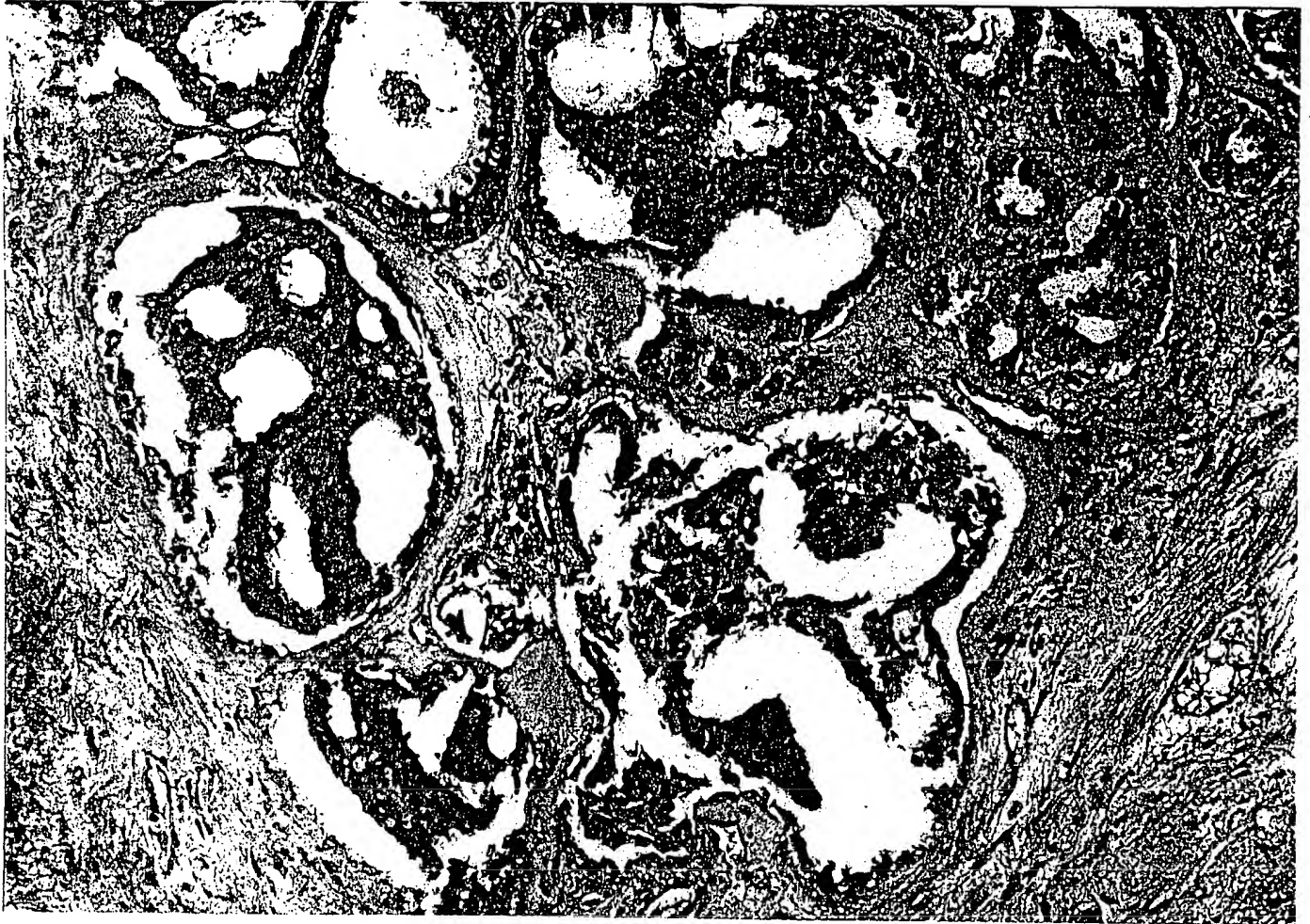


FIGURE 23

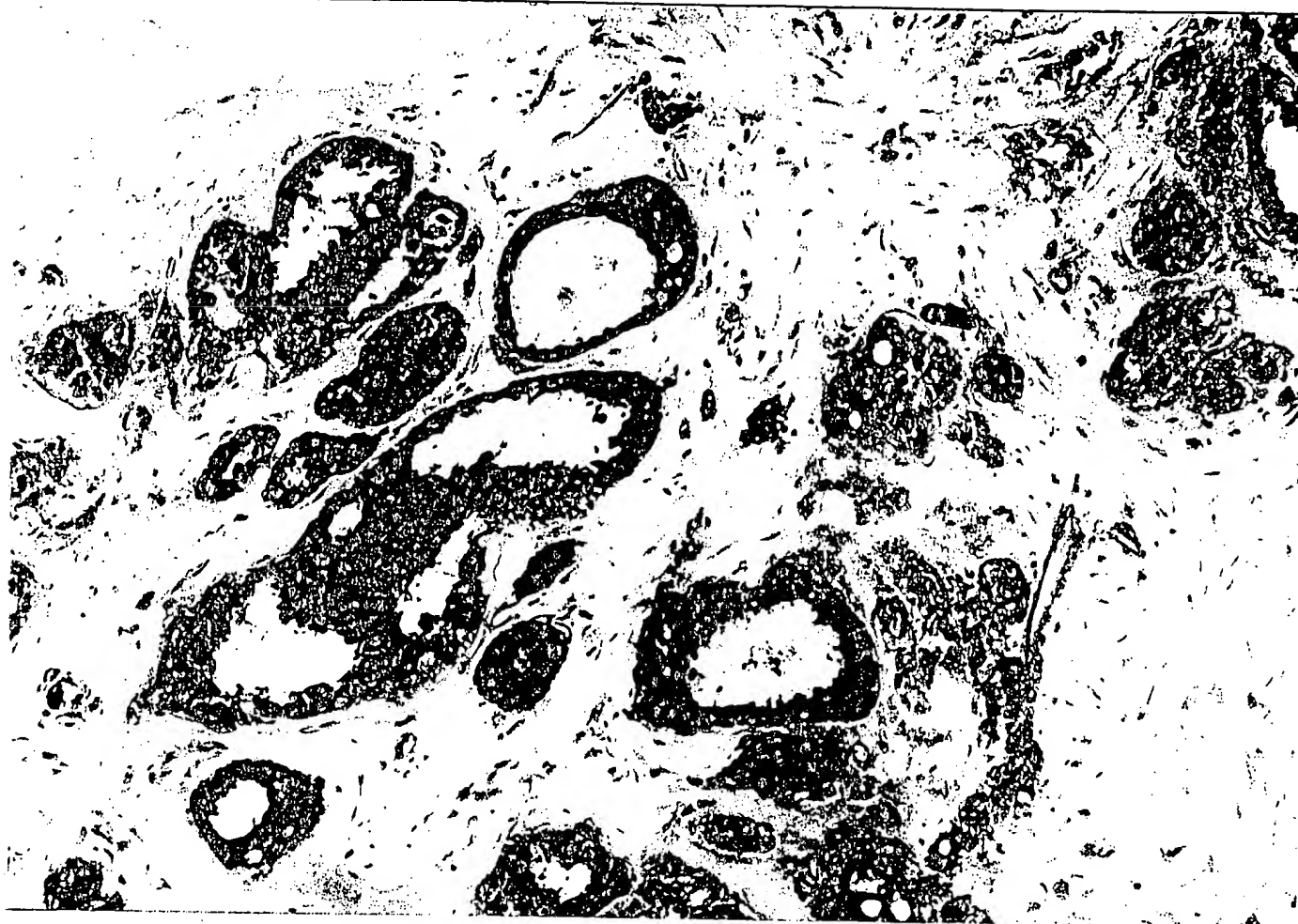
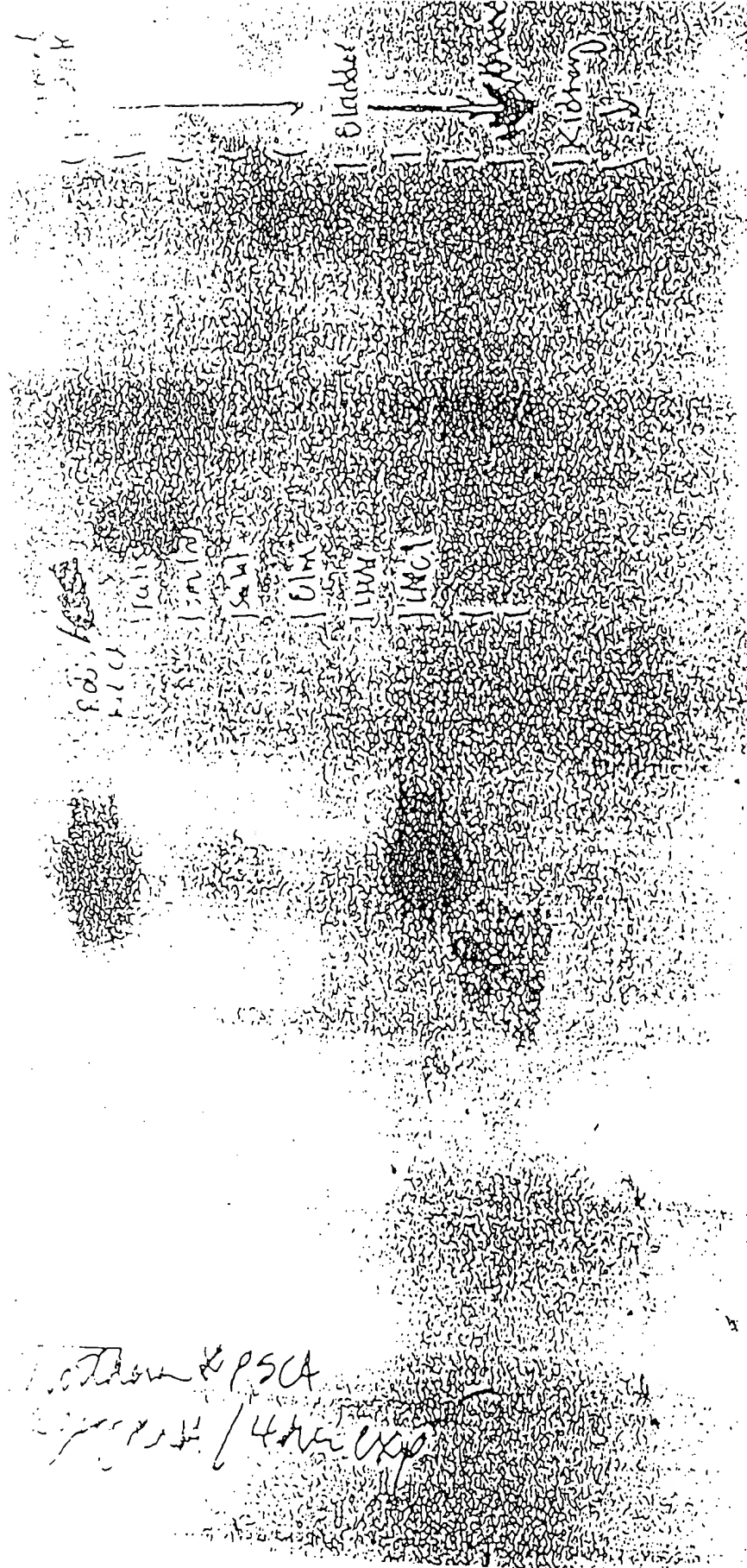


FIGURE 24



To: Thomas & PSCA
From: [illegible] / 4th floor

FIGURE 25

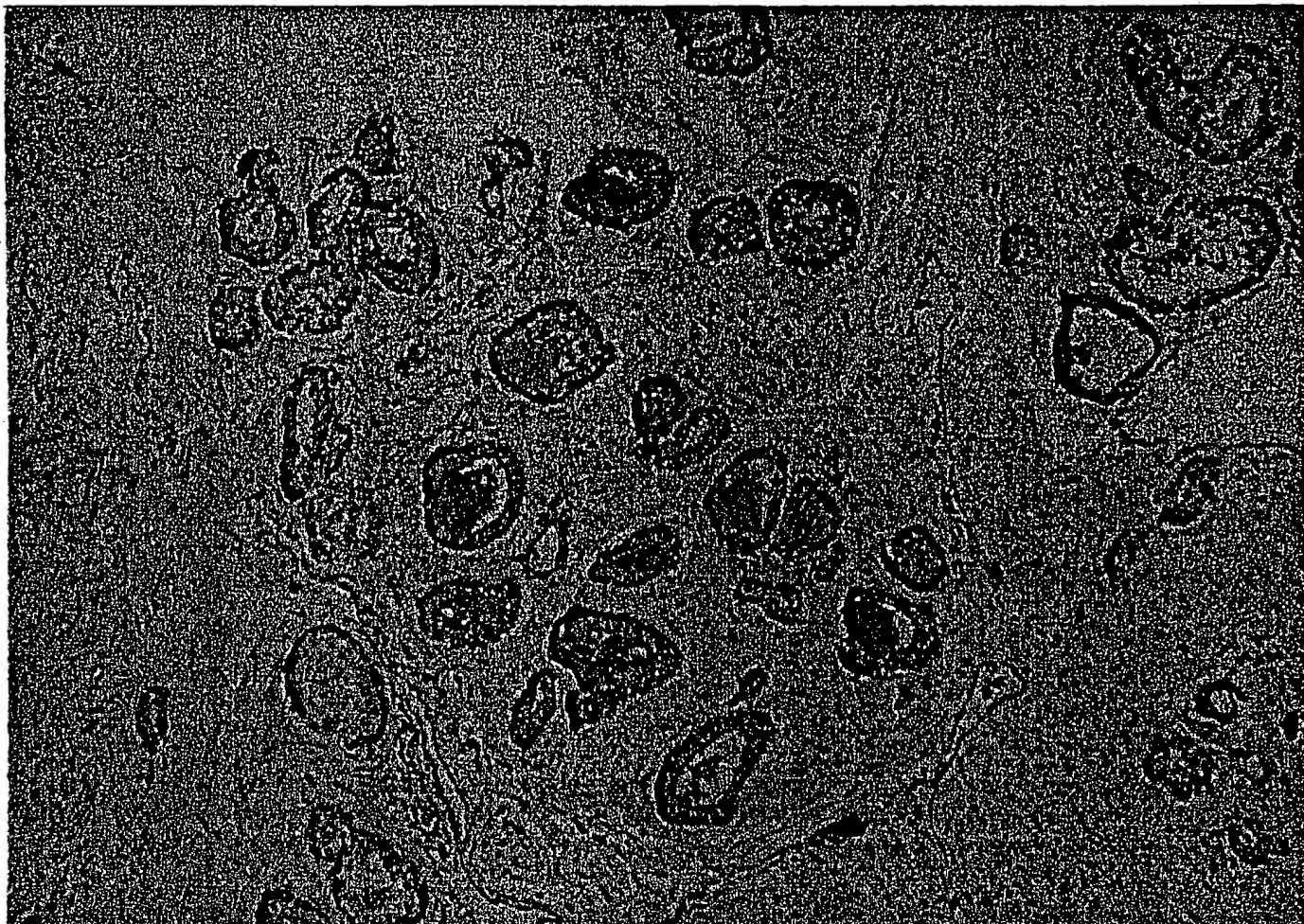


FIGURE 26

U.S. DEPARTMENT OF
HEALTH, EDUCATION &
WELFARE
NATIONAL INSTITUTE OF
ENVIRONMENTAL HEALTH
SCIENCES
RESEARCH TRIANGLE PARK,
NORTH CAROLINA 27709

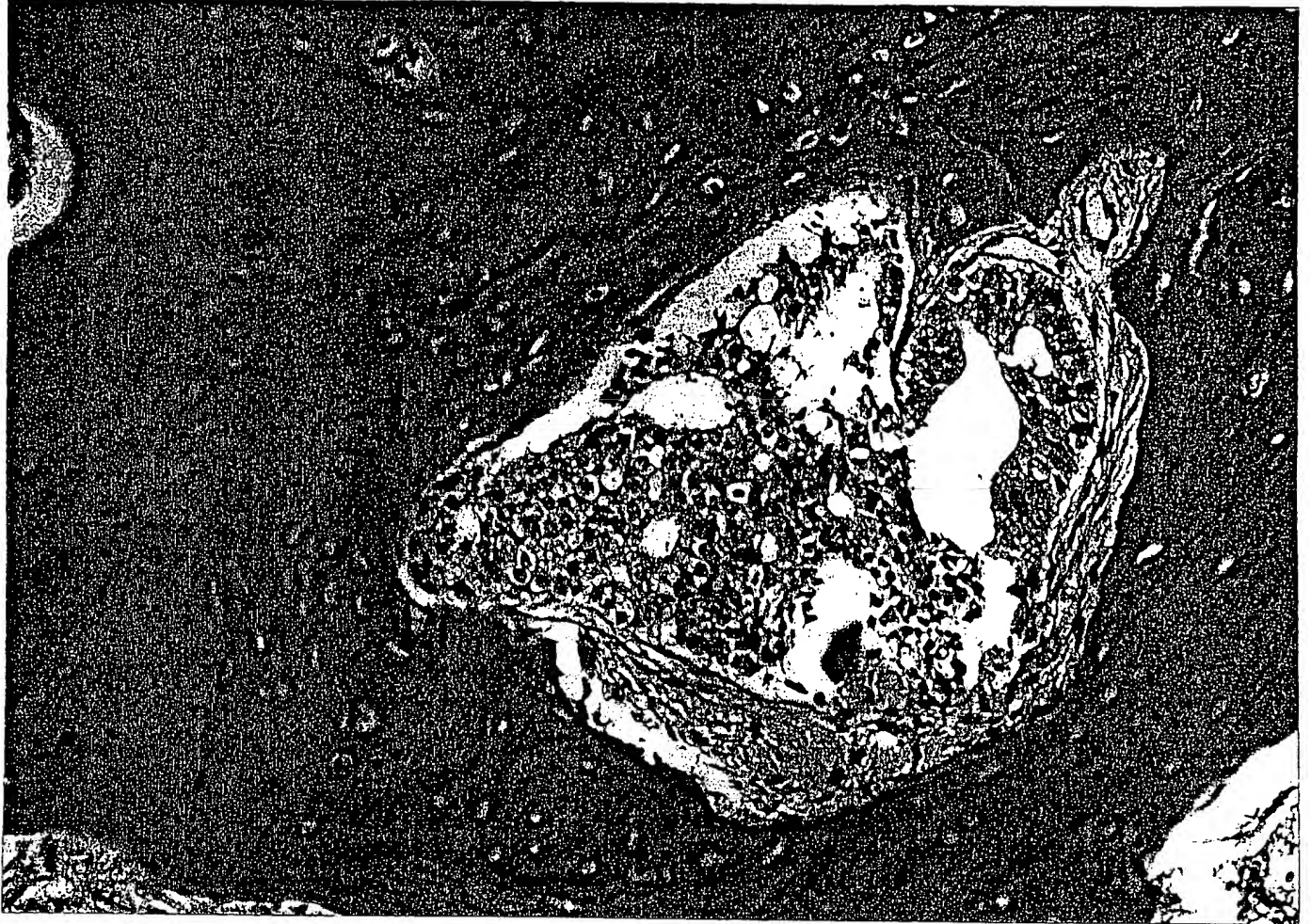
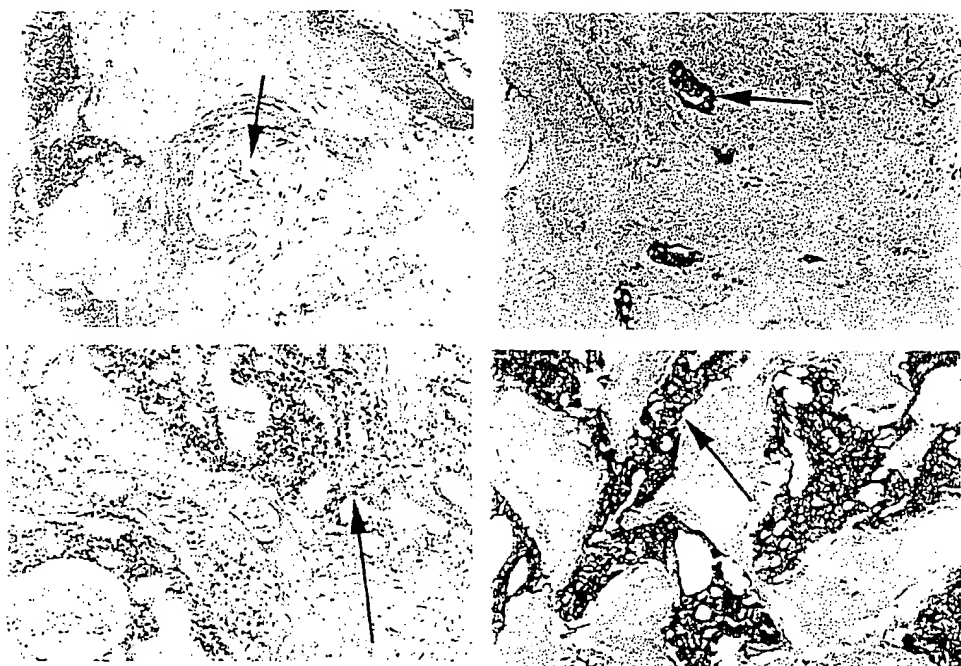


FIGURE 27



PSCA Immunostaining of Bony Metastases



Patient 5: H and E
and mAb 1G8

Patient 4: H and E
and mAb 3E6

FIGURE 28

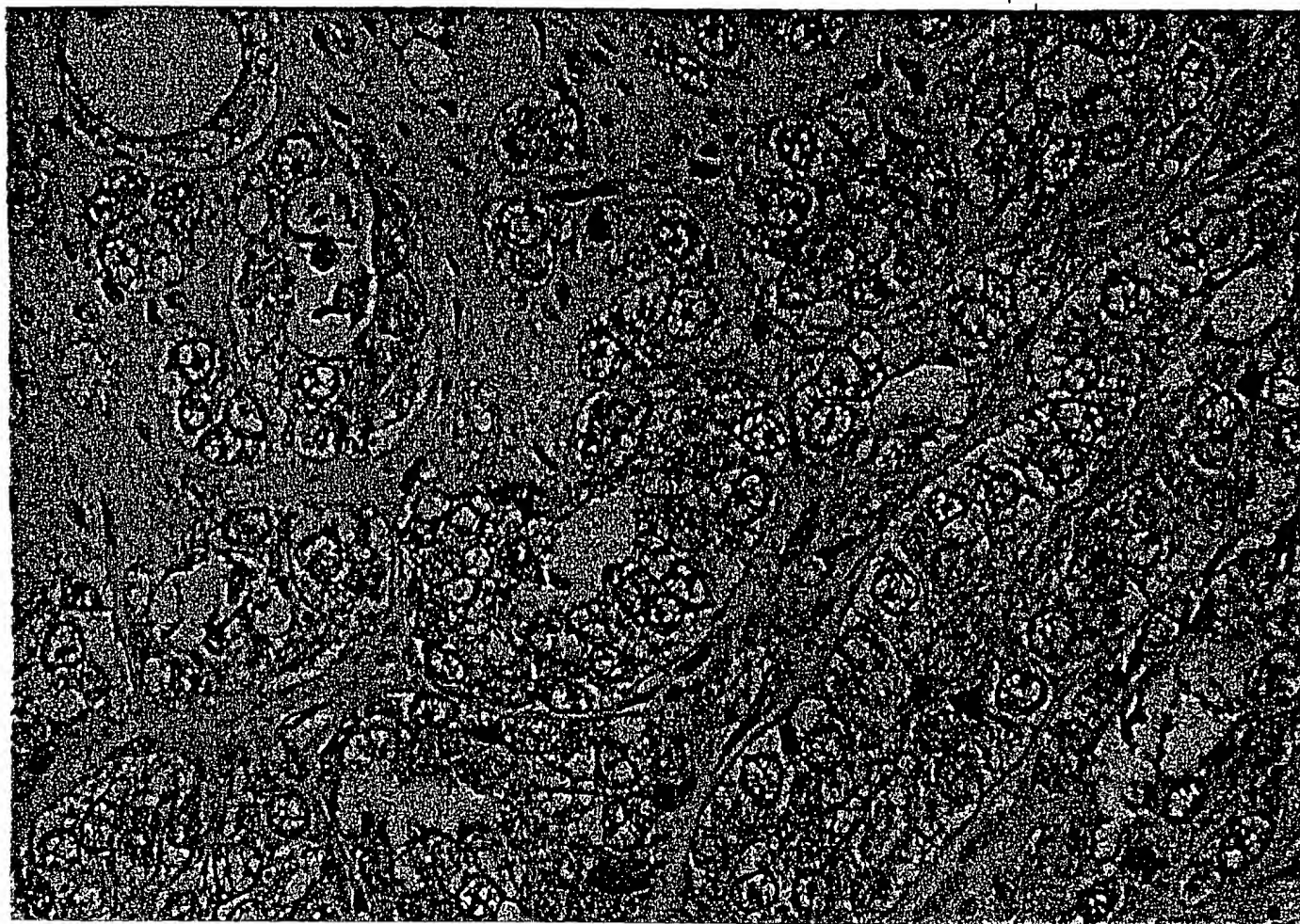


FIGURE 29

RECEIVED
JUL 10 1964
PHARMACY

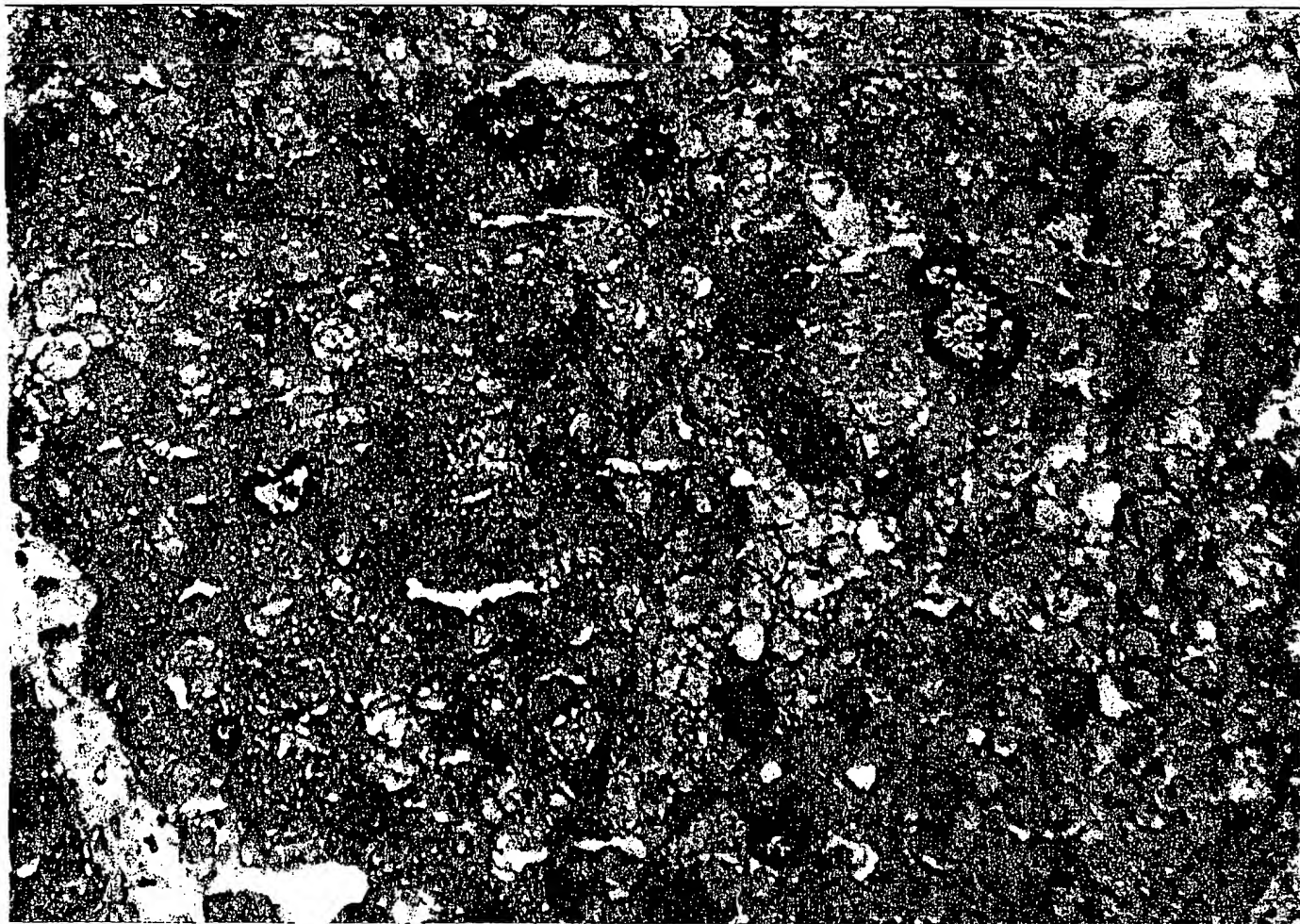


FIGURE 30

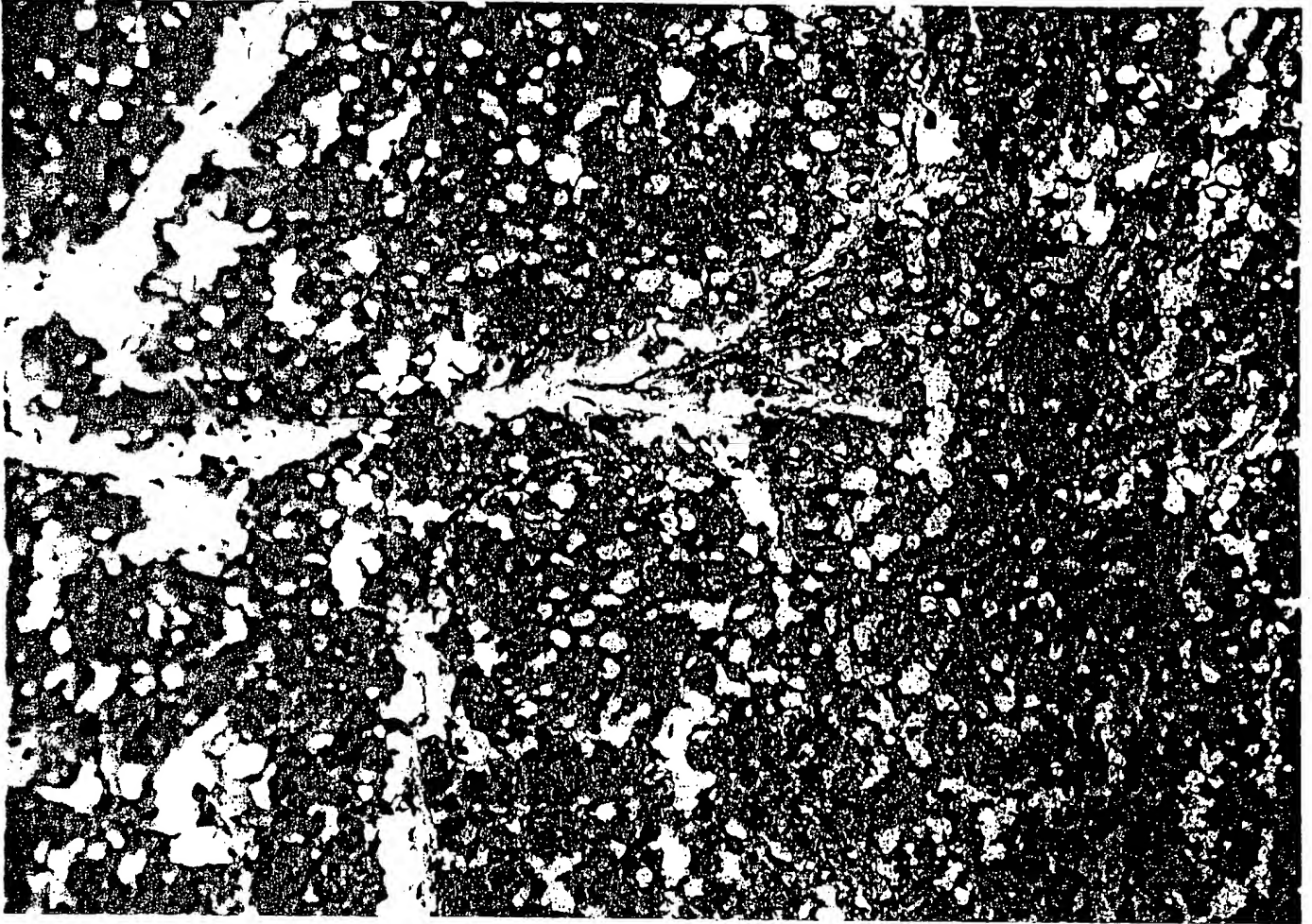


FIGURE 31

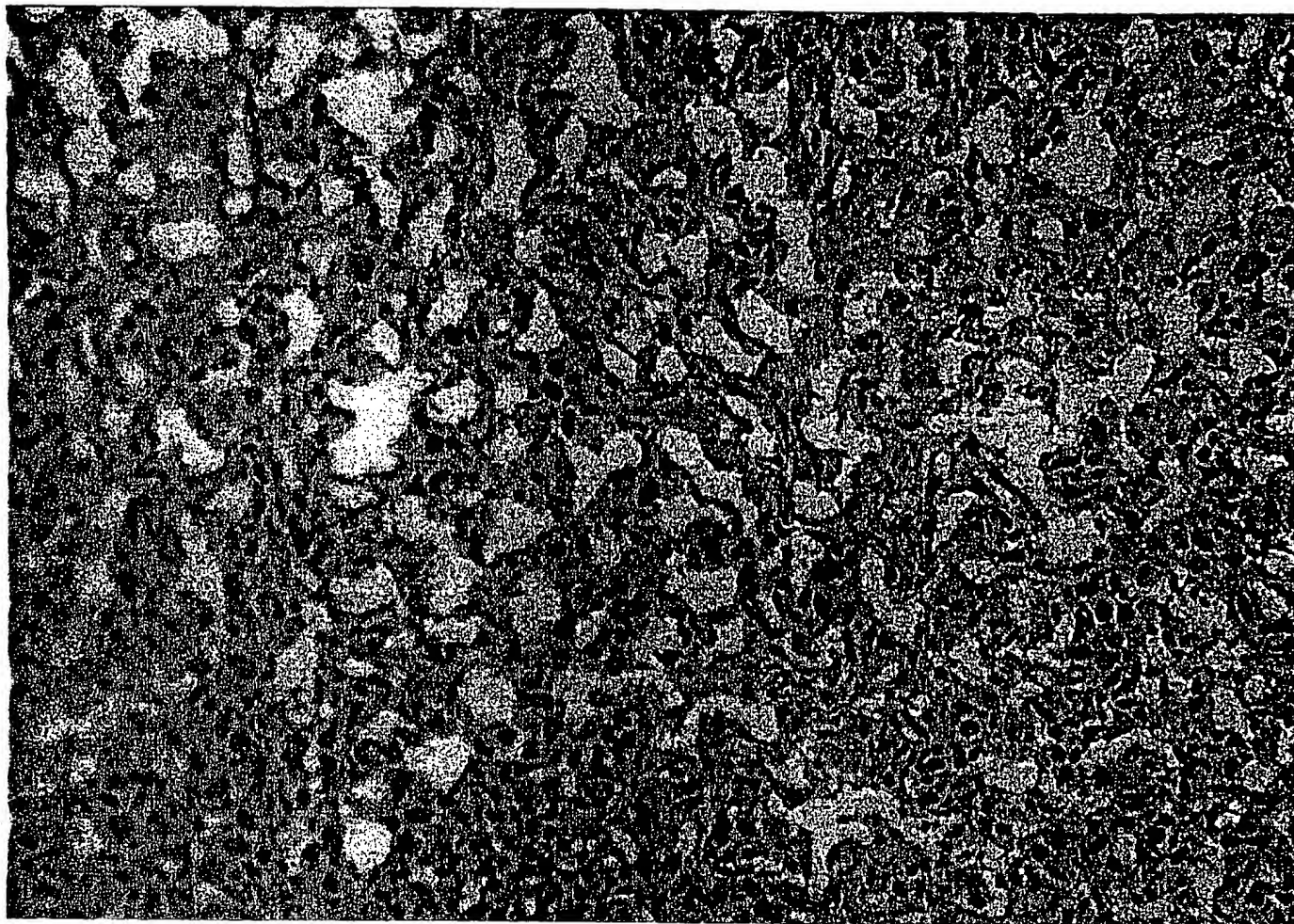
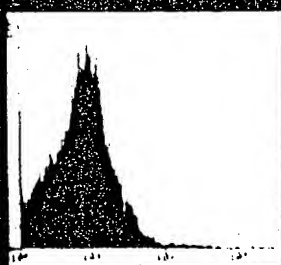


FIGURE 32

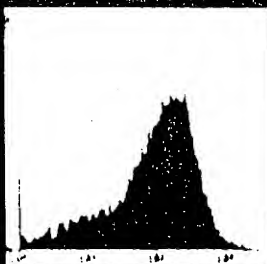


PSCA Expression in LAPC-9 Xenograft by FACS

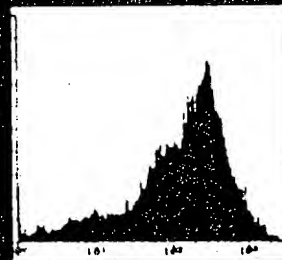
Secondary Antibody



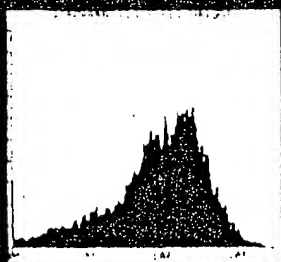
1G8



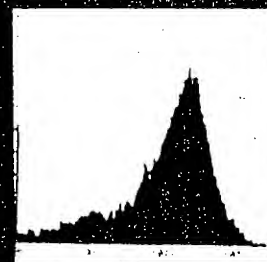
2H9



4A10



3C5



3E6

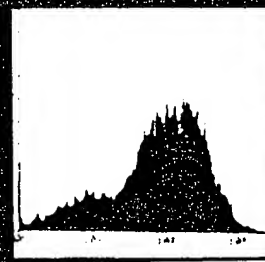


FIGURE 33

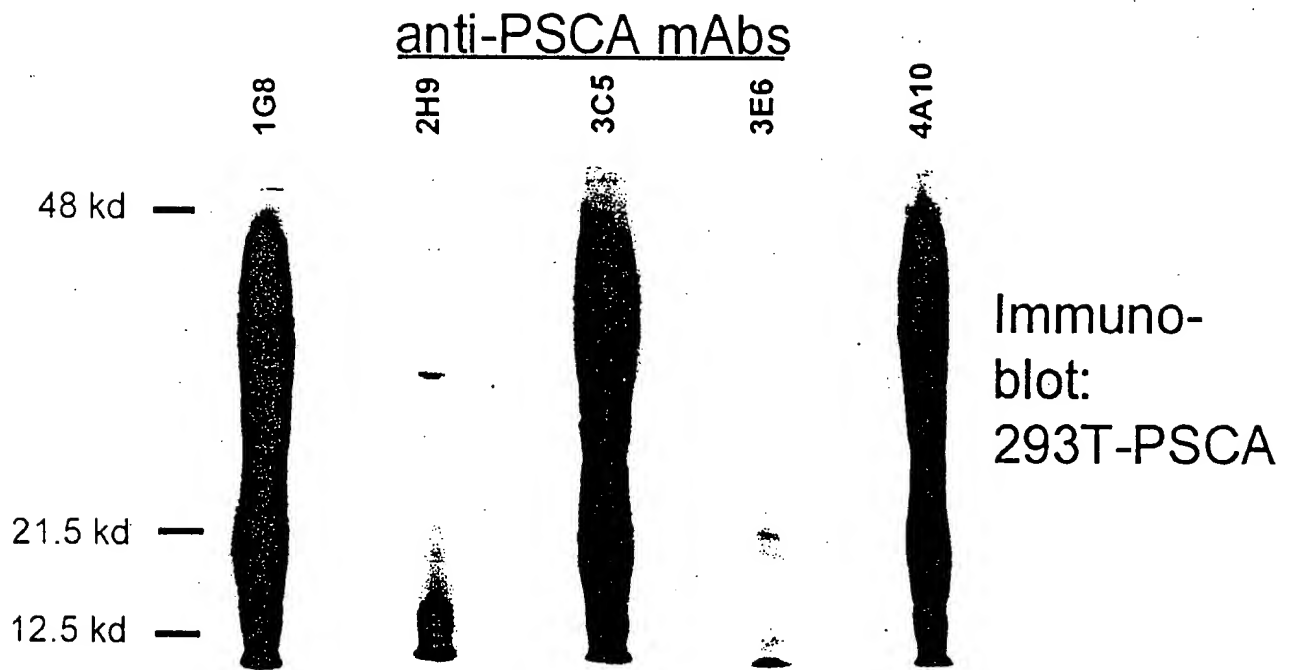


FIGURE 34



Immunofluorescent Staining of LNCaP-PSCA Cells

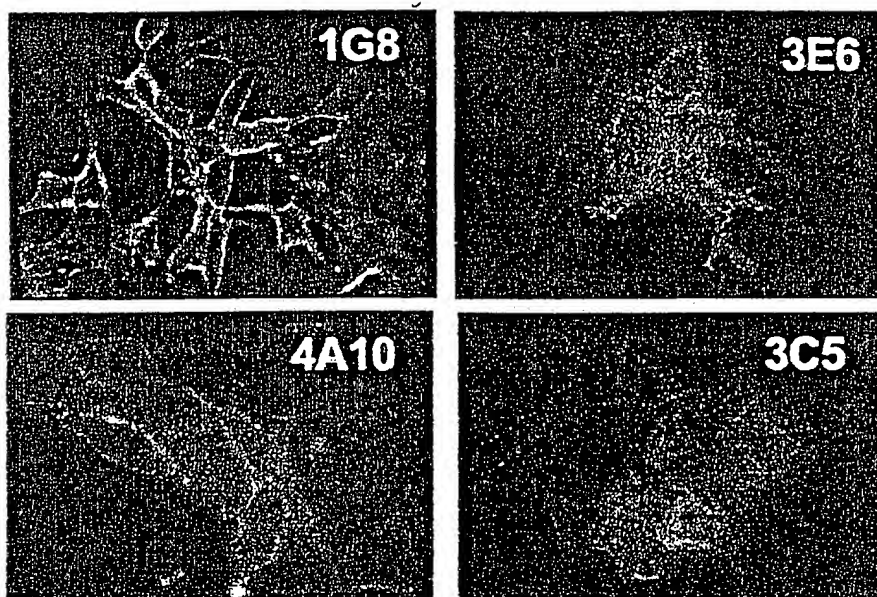


FIGURE 35



FIGURE 36

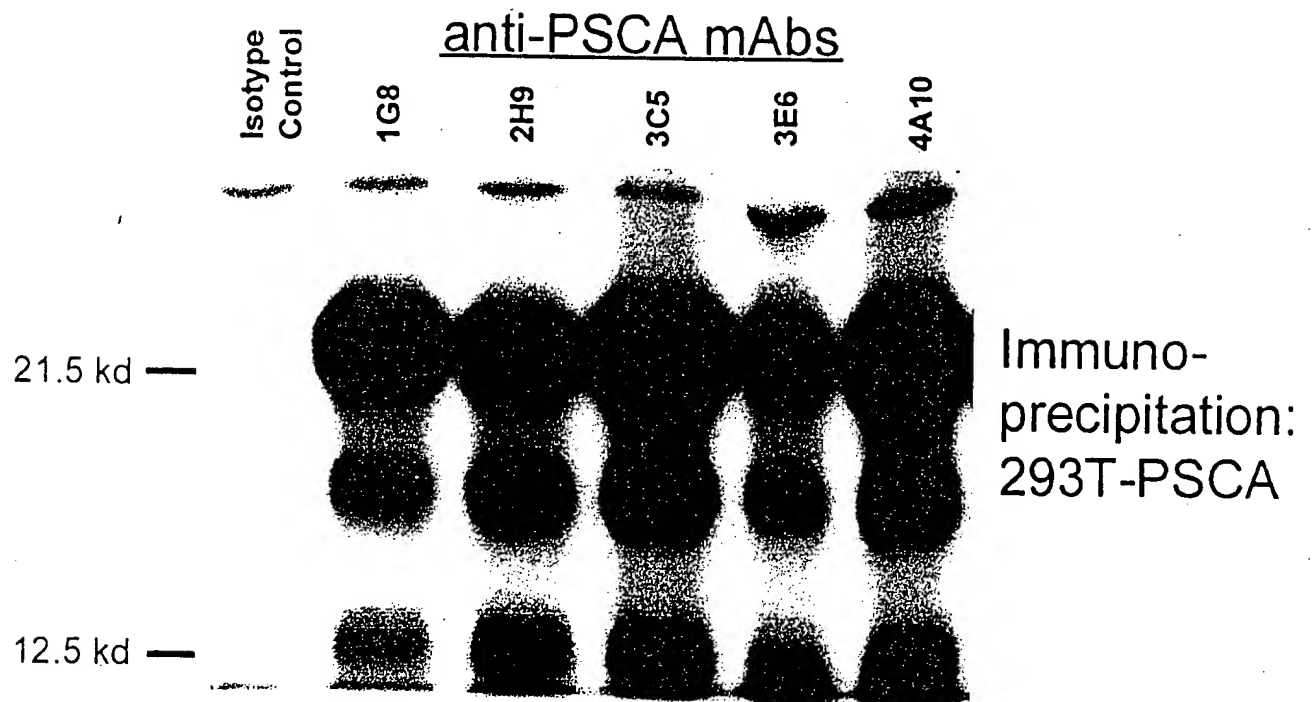
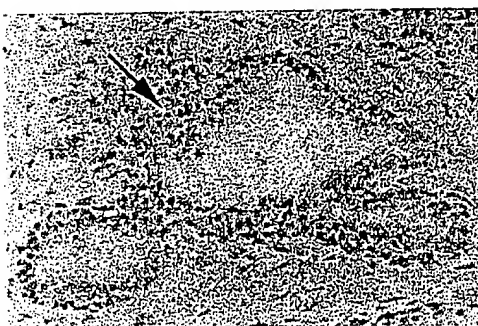


FIGURE 37



Immunohistochemical Staining of Normal Prostate

Normal: Isotype Control



Normal: PSCA mAb 3E6



Normal: PSCA mAb 1G8



Atrophy: PSCA mAb 2H9

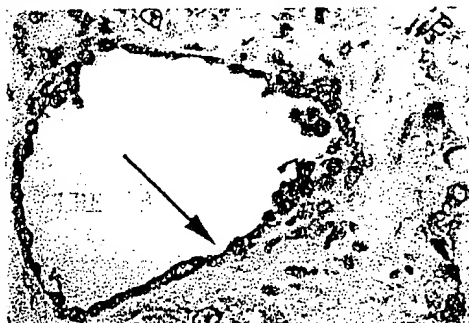
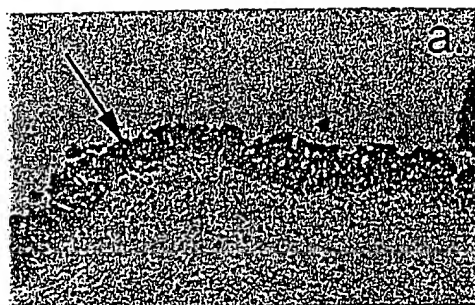
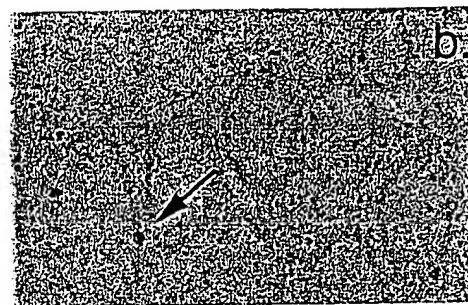


FIGURE 38

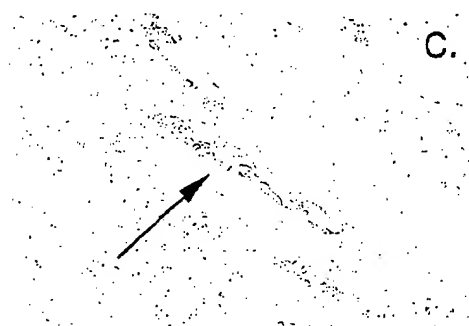
A.



Bladder: 1G8



Colon: 1G8



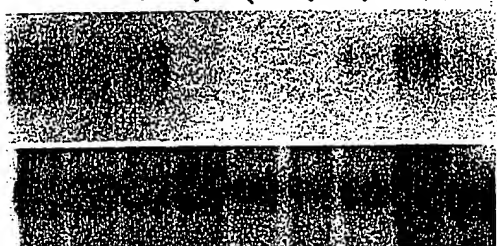
Kidney: 3E6



Placenta: 3E6

B.

Prostate
Prostate
Prostate
Kidney
Kidney
Kidney
Bladder
Bladder
Bladder
LAPC 9

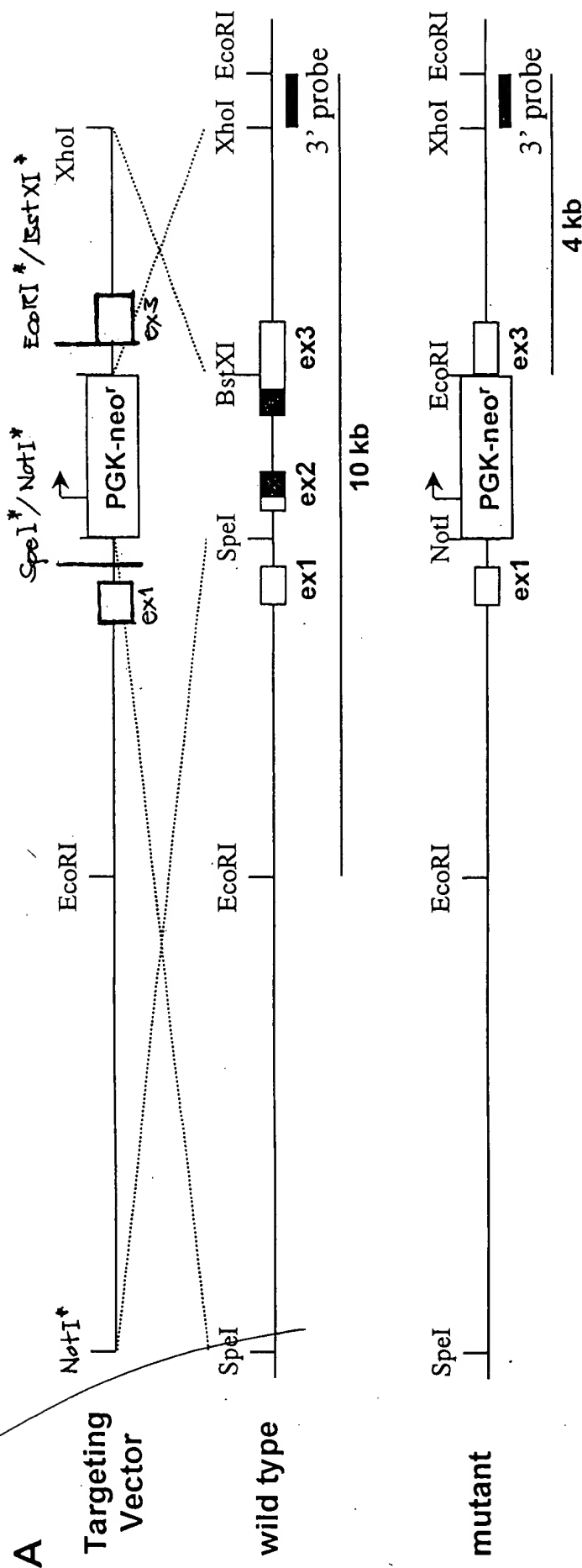


PSCA

Actin

FIGURE 39

Targeting of Mouse PSCA Gene



B. Genomic Southern Analysis of ES Cells

- ex1, 2, and 3 are the exons of PSCA gene.
- Black boxes of ex2 and ex3 encode PSCA mature protein sequences.
- ES genomic DNAs were digested with EcoRI, followed by Southern hybridization using 3' probe.

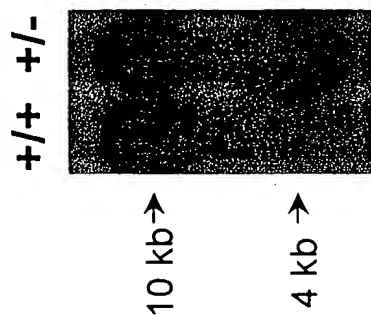
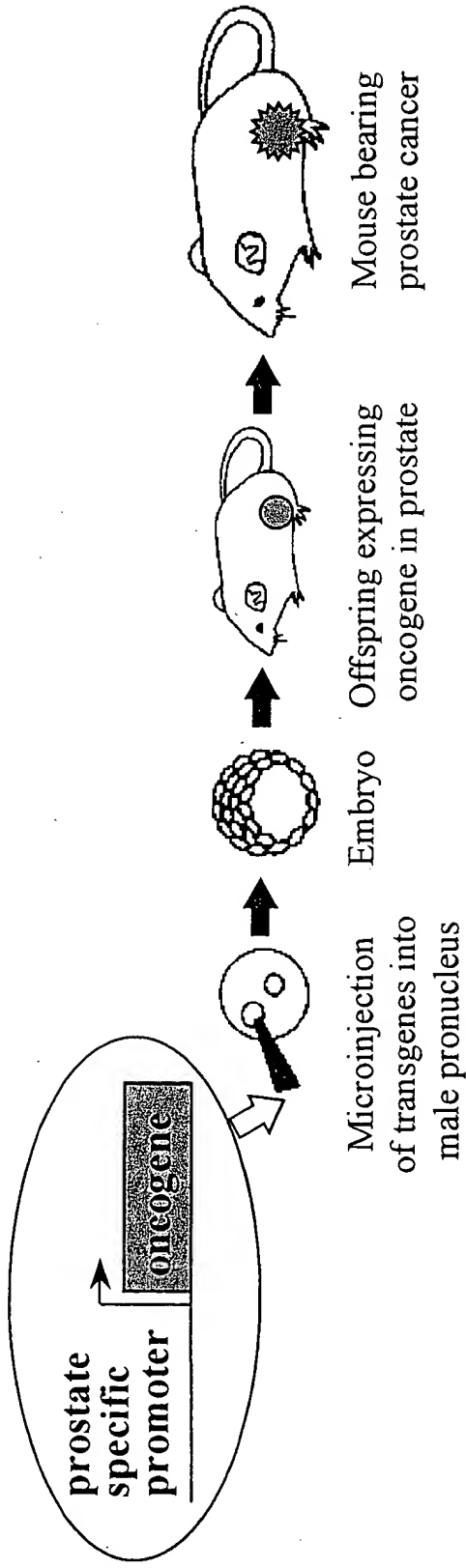


FIGURE 40

Transgenic Mouse Models of Prostate Cancer



Transgene	Target tissues	Characteristics
C3(1) (-3 kb)/ SV40 large+small, T <i>Maroulakou et al.</i> 1994 <i>PNAS</i>	prostate (secretory cells) urethral, mammary and sweat gland	Low-grade PIN 8-12 wks High-grade PIN 8-12 wks Invasive carcinoma 28 wks No metastases
Probasin (-426 bp)/ SV40 large+small, T <i>Greenberg et al.</i> 1995 <i>PNAS</i>	prostate (secretory cells)	Low-grade PIN 5-8 wks High-grade PIN 8-12 wks Invasive carcinoma 12 wks Metastases in lymph node, lung, liver and bone
Cryptdin2 (-6.5 kb)/ SV40 large+small, T <i>Garabedian et al.</i> 1998 <i>PNAS</i>	prostate (neuroendocrine cells) small intestine	Low-grade PIN 8-12 wks High-grade PIN 8-12 wks Invasive carcinoma 16 wks Metastases in lymph node, lung, liver and bone



Reporter Gene Constructs for Transfection Assay

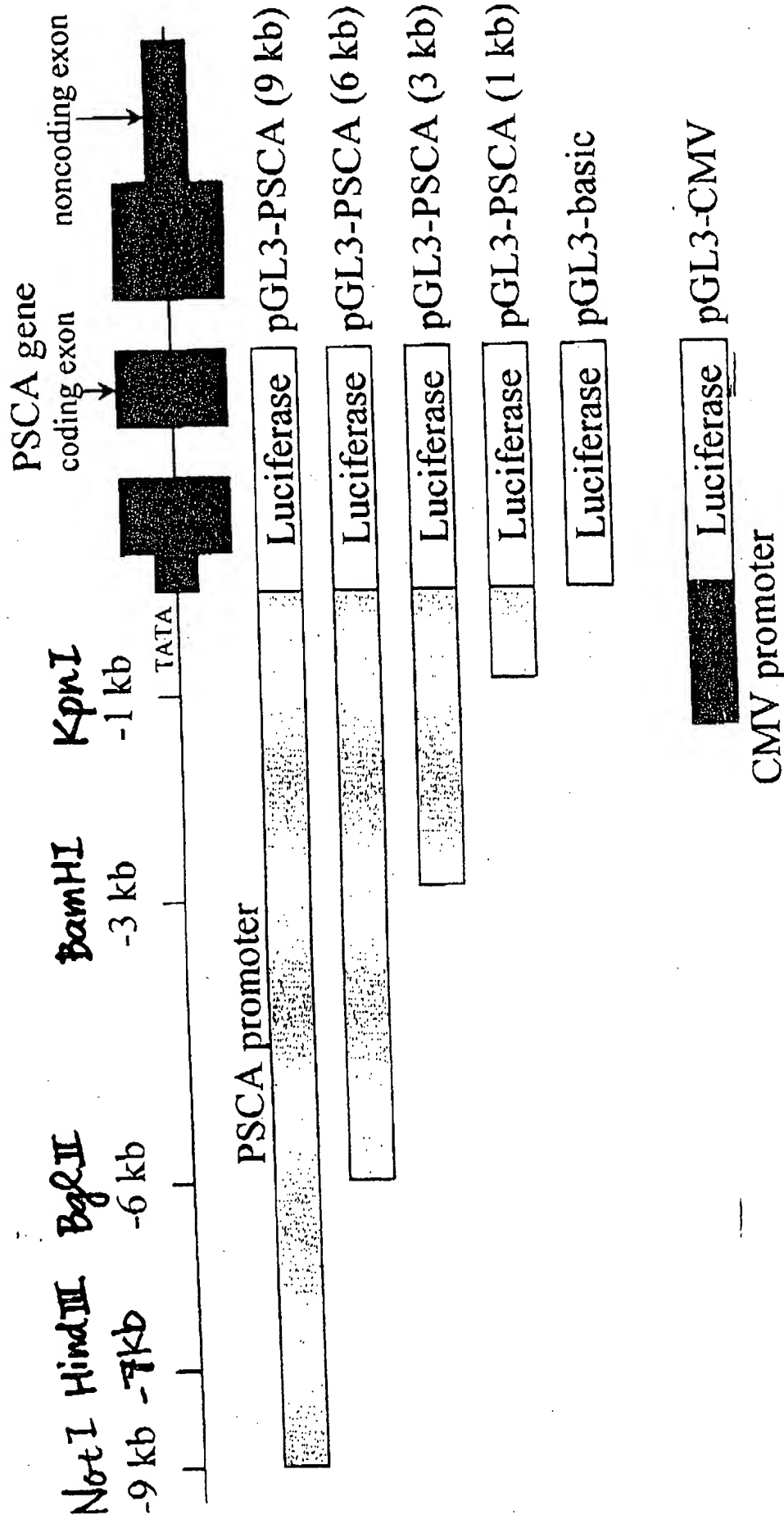


FIGURE 42

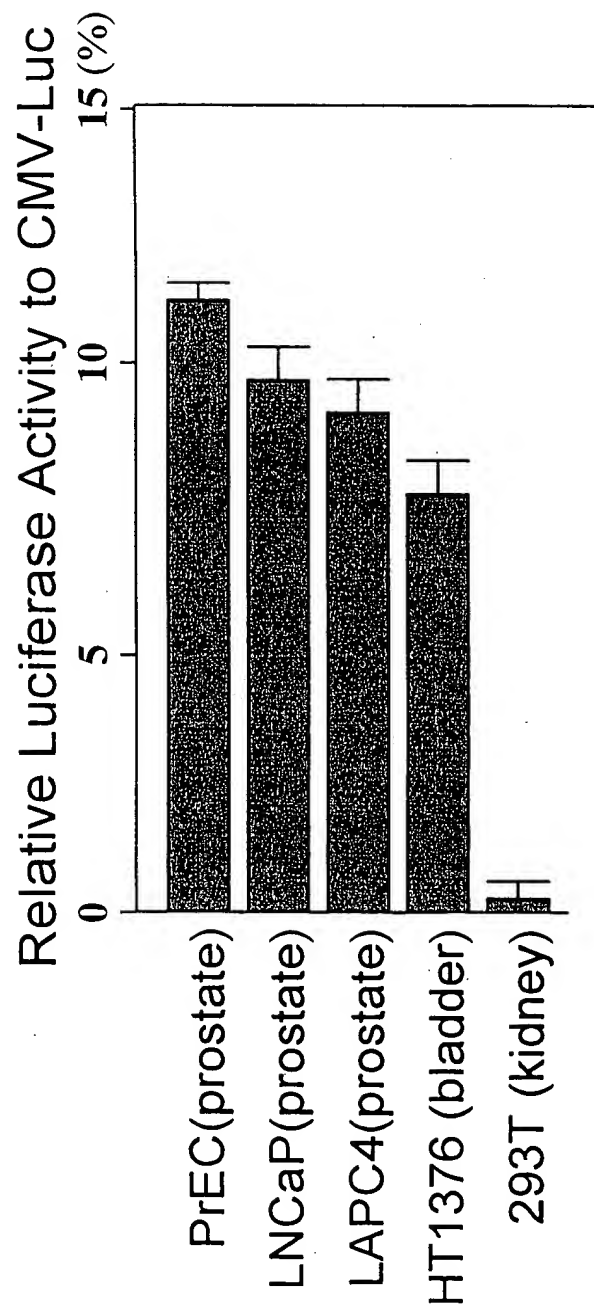


FIGURE 43



Identification of Prostate-Specific Elements Within PSCA Promoter Sequences

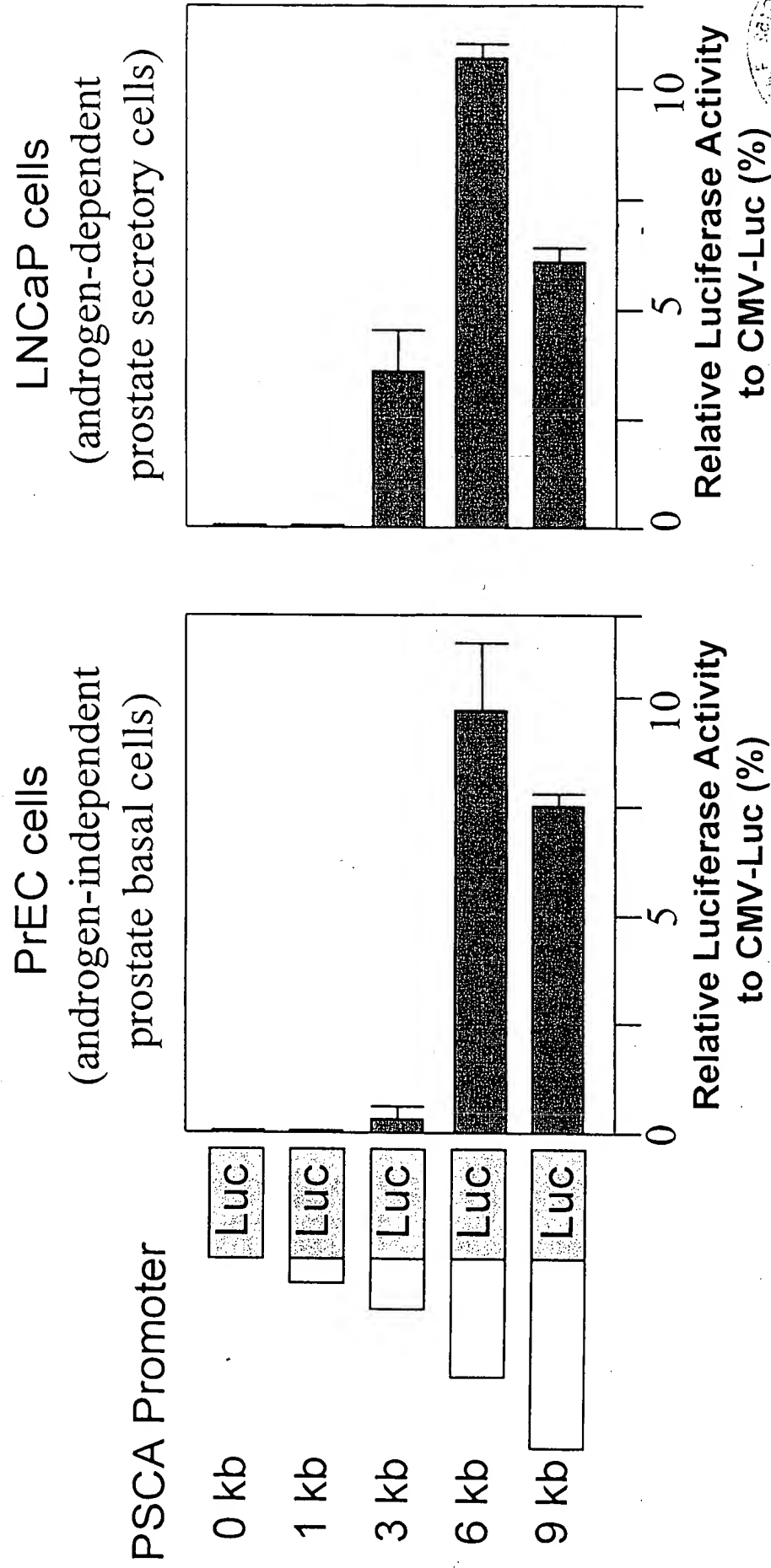


FIGURE 44

Update of Transgenic Mouse Projects

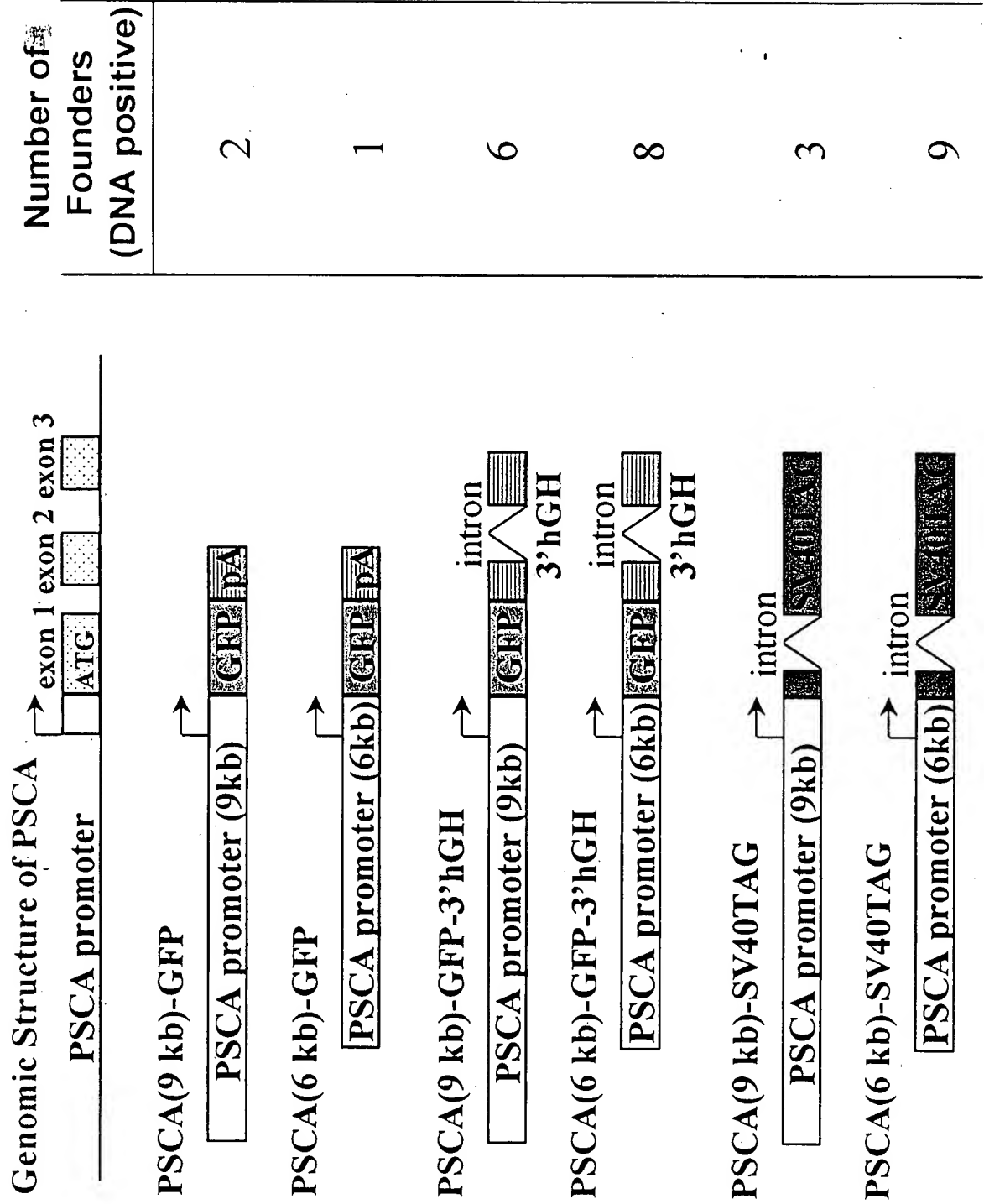


FIGURE 45



Whole-mount green fluorescence image

	Transgenic	Non-transgenic
Prostate (A25-106-2)		
Bladder (A25-104)		
Skin (A25-106-2)		

Negative tissues

Stomach
Small intestine
Colon
Seminal Vesicle
Urethra
Testis
Liver
Kidney
Lung
Brain
Heart
Skeletal muscle
Ovary
Uterus

HUMAN

Spleen
Thymus
Prostate
Testis
Ovary
S. int.
Colon
PBL

Heart
Brain
Placenta
Lung
Liver
Muscle
Kidney
Panc.

hPSCA →

Northern Analysis

MOUSE

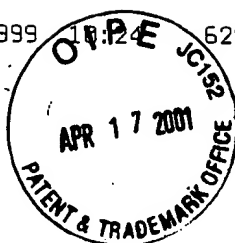
Ant. prostate
Dorso/Lat. prostate
Bladder prostate
Semin. vesicle
Urethra
Testis
Kidney
Esophagus
Body of stomach
Pyloric stomach
Duodenum
Small intestine
Colon
Salivary gland
Spleen
Thymus
Bone marrow
Skeletal muscle
Heart
Brain
Eye
Lung
Liver
Skin

mPSCA →

mG3PDH →

RT-PCR

FIGURE 47



MANDEL & ADRIANO

United States Patent Application

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: PSCA: PROSTATE STEM CELL ANTIGEN AND USES THEREOF

The specification of which

- a. ☒ is attached hereto
- b. ☐ was filed on _____ as application serial no. _____ and was amended on _____ (if applicable) (in the case of a PCT-filed application) described and claimed in international no. _____ filed _____ and as amended on _____ (if any), which I have reviewed and for which I solicit a United States patent.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56 (attached hereto).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on the basis of which priority is claimed:

- a. ☐ no such applications have been filed.
- b. ☐ such applications have been filed as follows:

FOREIGN APPLICATION(S), IF ANY, CLAIMING PRIORITY UNDER 35 USC § 119			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)
ALL FOREIGN APPLICATION(S), IF ANY, FILED BEFORE THE PRIORITY APPLICATION(S)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)

I hereby claim the benefit under Title 35, United States Code, § 120/365 of any United States and PCT international application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. APPLICATION NUMBER	DATE OF FILING (day, month, year)	STATUS (patented, pending, abandoned)
09/038,261	March 10, 1998	Pending
09/203,939	December 2, 1998	Pending
09/251,835	February 17, 1999	Pending

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

U.S. PROVISIONAL APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)
60/814,279	March 10, 1997
60/071,141	January 12, 1998
60/074,675	February 13, 1998

I hereby appoint the following attorney(s) and/or patent agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith:

Sarah B. Adriano Reg. No. 34,470
SaraLynn Mandel Reg. No. 31,853

I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Mandel & Adriano to the contrary.

Please direct all correspondence in this case to Mandel & Adriano at the address indicated below:

Mandel & Adriano
35 No. Arroyo Parkway, Suite 60
Pasadena, California 91103

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2	Full Name Of Inventor	Family Name Reiter	First Given Name Robert	Second Given Name E.
0	Residence & Citizenship	City Los Angeles	State or Foreign Country California	Country of Citizenship US
1	Post Office Address	Post Office Address 10511 Kinnard Avenue	City Los Angeles	State & Zip Code/Country California 90024-6017/USA
Signature of Inventor 201: <i>Robert E. Reiter</i>				Date: <i>May 20 1998</i>
2	Full Name Of Inventor	Family Name Witte	First Given Name Owen	Second Given Name N.
0	Residence & Citizenship	City Sherman Oaks	State or Foreign Country California	Country of Citizenship US
2	Post Office Address	Post Office Address 14727 Sutton Street	City Sherman Oaks	State & Zip Code/Country California 91403/USA
Signature of Inventor 202: <i>Owen M. Witte</i>				Date: <i>May 20 1998</i>

§ 1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any

existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced, attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

- (1) prior art cited in search reports of a foreign patent office in a counterpart application, and
 - (2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.
- (b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and
- (1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or
 - (2) It refutes, or is inconsistent with, a position the applicant takes in:
 - (i) Opposing an argument of unpatentability relied on by the Office, or
 - (ii) Asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

- (c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:
- (1) Each inventor named in the application;
 - (2) Each attorney or agent who prepares or prosecutes the application; and
 - (3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.
- (d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.



RECORDATION FORM COVER SHEET PATENTS ONLY

U. S. DEPARTMENT OF COMMERCE

Patent and Trademark Office

To the Commissioner of Patents and Trademarks: Please record the attached original documents or copy thereof.

1. Name of conveying party(ies):

Robert E. Reiter
Owen N. Witte

2. Name and address of receiving party(ies):

The Regents of the University of California
1111 Franklin Street, 12th Floor
Oakland, California 94607-5200

Additional name(s) of conveying party(ies) attached? ☐ Yes ☐ No

Additional name(s) & address(es) attached? ☐ Yes ☐ No

3. Nature of conveyance:

- ☒ Assignment ☐ Merger
☐ Security Agreement ☐ Change of Name
☐ Other:

Execution Date: May 21, 1999

4. Application number(s) or patent number(s): Not yet known

If this document is being filed together with a new application, the execution date of the application is: May 25, 1999

A. Patent Application No.(s)

B. Patent No.(s)

Additional numbers attached? ☐ Yes ☐ No

5. Name and address of party whom correspondence concerning 6. Total number of applications and patents involved: 1 documents should be mailed:

Name: Sarah B. Adriano
Address: 35 N. Arroyo Parkway, Suite 60
Pasadena, California 91103

7. Total fee (37 CFR 3.41): \$40.00
☒ Enclosed
☐ Authorized to be charged to deposit account

8. Please charge any additional fees or credit any overpayments to our Deposit account number: 50-0306

DO NOT USE THIS SPACE

9. Statement and signature:

To the best of my knowledge and belief, the foregoing information is true and correct and any attached copy is a true copy of the Original document.

Sarah B. Adriano
Name of Person Signing

Sarah B. Adriano
Signature

5/25/99
Date

Total number of pages including cover sheet, attachments, and document:

Do not detach this portion

Mail documents to be recorded with required cover sheet information to:

Commissioner of Patents and Trademarks
Box Assignments
Washington, D.C. 20231

Public burden reporting for this sample cover sheet is estimated to average about 30 minutes per document to be recorded, including time for reviewing the Document and gathering the data needed, and completing and reviewing the sample cover sheet. Send comments regarding this burden estimate to the U.S. Patent and Trademark Office, Office of information systems, PK2-1000C, Washington, D.C. 20231, and to the Office of Management and Budget, Paperwork Reduction Project (0651-0011), Washington, D.C. 20503.

ASSIGNMENT

UCLA Case No. LA97-575-7

For good and valuable consideration, the receipt of which is hereby acknowledge,
ASSIGNOR (S),

1. Robert E. Reiter

2. Owen N. Witte

hereby sell, assign and transfer to ASSIGNEE, The Regents of the University of California, a California corporation, having its statewide administrative offices located at 1111 Franklin Street, 12th Fl., Oakland, CA 94607-5200, and the successors, assigns and legal representatives of the ASSIGNEE all of its right, title and interest for the United States and its territorial possessions and in all foreign countries in and to, any and all improvements which are disclosed in the invention entitled:

PSCA: PROSTATE STEM CELL ANTIGEN AND USES THEREOF

and which is found in

- (a) _____ U.S. patent application executed _____, entitled as above and listing the above-named persons as inventors
- (b) X U.S. application serial no. not yet known, filed herewith
- (c) _____ U.S. Patent No.: _____, issued _____

and any legal equivalent thereof in a foreign country, including the right to claim priority and, in and to, all Letters Patent to be obtained for said invention by the above application or any continuation, division, continuation-in-part, extension or substitute thereof, and any reissue, reexamination or extension of said Letters patent and all rights under all International Conventions for the Protection of Industrial Property:

ASSIGNOR (S) hereby covenants that no assignment, sale agreement or encumbrance has been or will be made or entered into which would conflict with his assignment;

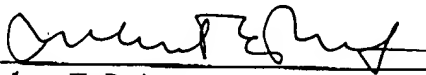
ASSIGNOR (S) further covenants that ASSIGNEE will, upon its request, be provided promptly with all pertinent facts and documents relating to said invention and said Letters Patent and legal equivalents as may be known and accessible to ASSIGNOR and will testify as to the same in any interference, litigation, or proceeding relating thereto and will promptly execute and deliver to ASSIGNEE or its legal representative any and all papers, instruments, or affidavits required to apply for, obtain, maintain, issue or enforce said application, said invention and said Letters Patent and said equivalents thereof which may be necessary or desirable to carry out the purposes thereof. An attorney of record is authorized and requested by the execution of this assignment to insert into this assignment the filing date and serial number of said application when officially known.

And the ASSIGNOR (S) requests the Commissioner of Patents and Trademarks to issue said Letters Patent of the United States and any reissue or extension thereof to the ASSIGNEE, The Regents of the University of California.

executed this

Signature of Inventor (s)

21 day of may, 1999


Robert E. Reiter

____ day of _____, 199_

Owen N. Witte

ASSIGNMENT

UCLA Case No. LA97-575-7

For good and valuable consideration, the receipt of which is hereby acknowledge,
ASSIGNOR (S),

1. Robert E. Reiter

2. Owen N. Witte

hereby sell, assign and transfer to ASSIGNEE, The Regents of the University of California, a California corporation, having its statewide administrative offices located at 1111 Franklin Street, 12th Fl., Oakland, CA 94607-5200, and the successors, assigns and legal representatives of the ASSIGNEE all of its right, title and interest for the United States and its territorial possessions and in all foreign countries in and to, any and all improvements which are disclosed in the invention entitled:

PSCA: PROSTATE STEM CELL ANTIGEN AND USES THEREOF

and which is found in

- (a) _____ U.S. patent application executed _____, entitled as above and listing the above-named persons as inventors
- (b) X U.S. application serial no. not yet known, filed herewith
- (c) _____ U.S. Patent No.: _____, issued _____

and any legal equivalent thereof in a foreign country, including the right to claim priority and, in and to, all Letters Patent to be obtained for said invention by the above application or any continuation, division, continuation-in-part, extension or substitute thereof, and any reissue, reexamination or extension of said Letters patent and all rights under all International Conventions for the Protection of Industrial Property:

ASSIGNOR (S) hereby covenants that no assignment, sale agreement or encumbrance has been or will be made or entered into which would conflict with his assignment;

ASSIGNOR (S) further covenants that ASSIGNEE will, upon its request, be provided promptly with all pertinent facts and documents relating to said invention and said Letters Patent and legal equivalents as may be known and accessible to ASSIGNOR and will testify as to the same in any interference, litigation, or proceeding relating thereto and will promptly execute and deliver to ASSIGNEE or its legal representative any and all papers, instruments, or affidavits required to apply for, obtain, maintain, issue or enforce said application, said invention and said Letters Patent and said equivalents thereof which may be necessary or desirable to carry out the purposes thereof. An attorney of record is authorized and requested by the execution of this assignment to insert into this assignment the filing date and serial number of said application when officially known.

And the ASSIGNOR (S) requests the Commissioner of Patents and Trademarks to issue said Letters Patent of the United States and any reissue or extension thereof to the ASSIGNEE, The Regents of the University of California.

executed this

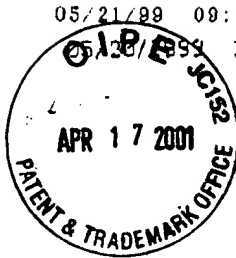
Signature of Inventor (s)

_____ day of _____, 199_

21ST day of May, 1999

Robert E. Reiter

Owen N. Witte
Owen N. Witte



NONPROFIT ORGANIZATION
VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 C.F.R. 1.9(e) AND 1.27(d)) • NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: The Regents of the University of California
 ADDRESS OF: 1111 Franklin Street, 12th Floor
 ORGANIZATION: Oakland, California 94607-5200

TYPE OF ORGANIZATION:

- a) ☒ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
 b) ☒ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. 501(a) AND 501(c)(3))
 c) ☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA -- (NAME OF STATE _____)
 (CITATION OF STATUTE _____)
 d) ☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. 501(a) AND 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA
 e) ☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA -- (NAME OF STATE _____)
 (CITATION OF STATUTE _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. 1.9(e) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in regard to the invention, entitled PSCA: PROSTATE STEM CELL ANTIGEN AND USES THEREOF by inventor(s) Robert E. Reiter and Owen N. Witte described in

- a) ☒ the specification filed herewith.
 b) ☐ provisional application serial no. _____, filed _____.
 c) ☐ non-provisional application serial no. _____, filed _____.
 d) ☐ patent no. _____, issued _____.

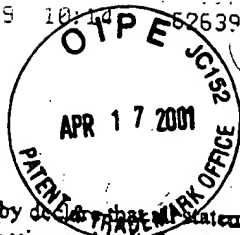
I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 C.F.R. 1.9(c) or by any concern which would not qualify as a small business concern under 37 C.F.R. 1.9(d) or a nonprofit organization under 37 C.F.R. 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 C.F.R. 1.27)

NAME UroGenesys
 ADDRESS 1701 Colorado Avenue Santa Monica, California 90404
 a) ☐ INDIVIDUAL b) ☒ SMALL BUSINESS CONCERN c) ☐ NONPROFIT ORGANIZATION

NAME _____
 ADDRESS _____
 a) ☐ INDIVIDUAL b) ☐ SMALL BUSINESS CONCERN c) ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. 1.28(b))



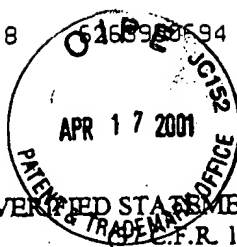
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereof, or any patent to which this verified statement is directed.

NAME: Linda Stevenson
TITLE: Principal Prosecution Analyst
ADDRESS: Office of Technology Transfer
University of California
Office of the President
1111 Franklin Street, 5th Floor
Oakland, California 94607-5200

SIGNATURE:

DATE:

May 21, 1999

**SMALL BUSINESS****VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 C.F.R. 1.9(f) AND 1.27(c)) - SMALL BUSINESS CONCERN**

I hereby declare that I am

- a) ☒ the founder of the small business concern identified below:
 b) ☐ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: UroGenesys
 ADDRESS OF CONCERN: 1701 Colorado Avenue
 Santa Monica, California 90404

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 C.F.R. 121.801-805, and reproduced in 37 C.F.R. 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled PSCA: PROSTATE STEM CELL ANTIGEN AND USES THEREOF by inventor(s) Robert E. Reiter and Owen N. Witte described in

- a) ☒ the specification filed herewith.
 b) ☐ provisional application serial no. _____, filed _____.
 c) ☐ non-provisional application serial no. _____, filed _____.
 d) ☐ patent no. _____, issued _____.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 C.F.R. 1.9(c) or by any concern which would not qualify as a small business concern under 37 C.F.R. 1.9(d) or a nonprofit organization under 37 C.F.R. 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 C.F.R. 1.27)

NAME The Regents of the University of California
 ADDRESS 1111 Franklin Street, 12th Floor, Oakland, California 94607-5200
 a) ☐ INDIVIDUAL b) ☐ SMALL BUSINESS CONCERN c) ☒ NONPROFIT ORGANIZATION

NAME _____
 ADDRESS _____
 a) ☐ INDIVIDUAL b) ☐ SMALL BUSINESS CONCERN c) ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereof, or any patent to which this verified statement is directed.

NAME: Donald B. Rice
 TITLE: President and CEO
 ADDRESS: 1701 Colorado Avenue, Santa Monica, California 90404

SIGNATURE: Donald B. RiceDate: 5/20/99